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THE EXPERIMENTAL PRODUCTION AND DEVELOPMENT OF DIFFUSE HEPATIC FIBROSIS (" PORTAL CIRRHOSIS ").

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IT is now securely established that, by means of abnormal diets, fatty infiltration, necrosis and " cirrhosis " of the liver can be produced in experimental animals. The majority of workers have regarded, and continue to regard, such dietetic injury as an entity in which fatty infiltration, necrosis and " cirrhosis " are but stages in one sequence. Daft, Sebrell and Lillie (1942) early dissented from this view and, on the basis of experiments showing that particular dietary supplements affected differently the incidence of necrosis and of fibrosis, suggested that these two conditions were distinct, but, being unable to produce one lesion independently of the other, their suggestions did not meet with general acceptance (Bollman, 1943). In 1944, however, Himsworth and Glynn claimed to have produced separately two distinct anatomical lesions by dietary means ; one a massive necrosis, the survivors of which developed post-necrotic scarring and nodular hyperplasia ; the other an insidiously developing diffuse hepatic fibrosis after fatty infiltration. Their view differed from that of Daft and his collaborators in that the distinction drawn was, not between necrosis and fibrosis, but between two sequences both of which ended in fibrosis and to only one of which necrosis is a necessary antecedent. The position with regard to the distinction of the massive necrosis—post-necrotic scarring sequence has since been further strengthened by showing that it can occur at any level of fatty infiltration of the liver (Glynn and Himsworth, 1945), that it is independent of the dietary content of fat or lipotropic factors (Himsworth and Glynn, 1944), but that it is closely correlated with the amount of cystine in the diet (Glynn, Himsworth and Neuberger, 1945). It is the purpose of this paper to produce further evidence that the fatty infiltration—diffuse, hepatic, fibrosis, sequence is equally distinct, not only morphologically, but aetiologically.

It so happens that the nutritive factors whose deficiency leads to one or other sequence tend to occur together in nature. Thus meat supplies, not only sulphur-

containing amino-acids but also lipotropic factors, such as choline (Best, Hershey and Huntsman, 1932; Best and Huntsman, 1932), which prevent fatty infiltration of the liver. Further, a factor which is essential for the prevention of the massive necrosis sequence may incidentally possess a general property which contributes to the prevention of the other. Methionine acts both as a source of cystine and of choline (Du Vigneaud, Dyer, Rachele and Cohn, 1945). As a consequence deficient diets constructed from crude food stuffs tend to produce mixed lesions with features of both the diffuse, hepatic fibrosis and the massive necrosis sequences. Such diets have been used by the majority of workers. It was early appreciated that fatty infiltration contributed in some way to the development of fibrotic lesions in the liver. The lipotropic action of protein was by then well established. It was only natural to test the effects of low-protein, high-fat diets; and the conspicuous lesions produced rapidly established the reputation of such. Inevitably such diets led indiscriminately to fatty infiltration, necrosis and fibrosis, and also to the unitary conception of dietetic injury.

At first sight it might appear that the matter could be tested by producing fatty infiltration on a high protein diet. But this is impossible as high protein diets prevent accumulation of fat in the liver. In an important paper Handler and Dubin (1946) attacked the problem in another way. They confirmed that a low protein-high fat diet produced fatty infiltration, necrosis, and fibrosis, and that the degree of fibrosis seemed, in general, to be related to the degree of fatty infiltration. They then investigated the effects of increasing the dietary casein, while preventing the lipotropic action of this high protein intake by an ingenious measure. The lipotropic action of casein is due to the methionine it contains, and the lipotropic action of methionine to its labile methyl group. By giving an excess of nicotinamide in the diet these methyl groups can be removed and the growth promoting and lipotropic actions of methionine abolished (Handler and Dann, 1942). Under these circumstances the moderate protein, high fat diet produced fatty infiltration, minimal necrosis, but no "cirrhosis." The inference was that even when fatty infiltration was thus artificially maintained at high levels, adequate dietary protein would prevent cirrhosis. But necrosis of a greater or less degree was reported for all their diets and consequently it is impossible to be certain whether the fibrosis observed was of the post-necrotic type, due to deficiency of sulphur-containing amino-acids, or of the diffuse hepatic fibrosis type, related to fatty infiltration, but the exact aetiology of which is still uncertain. If the mechanism of diffuse hepatic fibrosis is to be investigated then all possibility of confusion with post-necrotic scarring must be avoided. The diets used must never produce necrosis. The plan adopted in the experiments reported here was to give a diet containing just sufficient casein to protect against dietetic necrosis, to ensure further protection by supplements of cystine and of tocopherol (Schwartz, 1944; Gyorgy, 1947), each in amounts sufficient to protect alone, and to vary the fat and lipotropic content of the diet so as to produce different degrees of fatty infiltration of the liver. By these means a diffuse hepatic fibrosis, without any preceding necrosis, was produced and it was shown that the degree and speed of development of that fibrosis was proportional to the degree and duration of the preceding fatty infiltration, the nitrogen and caloric intake being constant in all experiments.

METHODS.

Animals.

Male albino rats of the Wistar strain from the Glaxo Laboratories colony were used. At the beginning of the experiment they weighed between 90 g. and 100 g. each. They were housed in groups of four in cages fitted with wire screen bottoms. 152 animals were used.

Diets.

Six diets were used and amounts of each were given to the respective groups of animals to ensure that in all groups the daily caloric, amino-acid, vitamin and salt intakes were identical. The variables were the fat, carbohydrate, and choline intakes and so, for convenience, the diets will be distinguished by their percentage content of fat and, when choline was given, the amount of the choline supplement. The detailed composition of the diets is given in Table I. The percentage of fat varied from 4 per cent, in diet F.4 per cent, to 50 per cent,

TABLE I.—*Composition of Diets.*

Food: mg./rat/day.

Diet No.	Calories.	Casein.	Cystine.	Salt.	Arachis oil.	Vitamins.	Lard.	Starch. Sugar-	Choline.
F.4 per cent	40	490	31	184	400	+	0	8560	0
F.16 per cent	,,	,,	,,	,,	,,	,,	920	6530	,,
F.30 per cent	,,	,,	,,	,,	,,	,,	1840	4520	,,
F.50 per cent	,,	,,	,,	,,	,,	,,	2760	2490	,,
F.50 per cent Ch.5	,,	,,	,,	,,	,,	,,	,,	,,	5
F.20 per cent Ch.20	,,	,,	,,	,,	,,	,,	,,	,,	20

The starch used was pure crushed maize starch; the sugar, sucrose.

in diet F.50 per cent, and for each increment of fat the dietary carbohydrate was reduced in isocaloric amounts. Two groups, taking the F.50 per cent diet, were given respectively 5 mg. and 20 mg./rat/day of choline and are distinguished as F.50 per cent Ch.5 and F.50 per cent Ch.20. The food was mixed in bulk and the arachis oil and the following vitamin supplements added to each daily portion; thiamine 25μ, riboflavin 20μ, pyridoxine 25μ, calcium pantothenate 100μ and cod liver oil two drops. Five mg. of α-tocopherol were given to each rat twice weekly.

Chemical methods.

These have been described (Glynn and Himsworth, 1945).

Histological methods.

The rats were killed by a blow on the head. Liver samples were fixed in 10 per cent formol saline and, after paraffin embedding, sections were cut at 6 μ. Ehrlich's acid haemotoxylin and eosin, Laidlaw's reticulin stain (Laidlaw, 1929) and the Van Gieson stain for fibrous tissue, were used.

RESULTS.

Histological Changes.

The sequence of histological changes will be described in detail elsewhere (Glynn and Himsworth, unpublished). The insidious development of a diffuse hepatic fibrosis from a heavy, prolonged, fatty infiltration was followed step by step. At no time was there evidence of frank necrosis. The stages in the process were arbitrarily graded as follows :

0 = fatty infiltration alone.
1 = thickening of reticulin fibres round the central veins with prolongations beginning to stretch out to adjoining central veins.
2 = linking of central veins by definite bands of reticulin with further prolongation beginning to stretch to the portal tracts.
3 = central veins and portal tracts linked together by conspicuous bands of reticulin which are beginning to mature into fibrous tissue as shown by the Van Gieson stain. At this stage the lobular pattern has disappeared and the parenchyma lies as angular masses in the fibrous network. Fatty infiltration is diminishing.
4 = the fibrous network is thicker and more mature ; the angular fragments of parenchyma have, by concentric hyperplasia, become round nodules ; fatty infiltration is disappearing.

Fatty Infiltration and Diffuse Hepatic Fibrosis.

The rapidity of development, and the degree, of fatty infiltration of the liver produced by the different diets was approximately proportional to the amount of fat these contained (Fig. 1). The results confirm those of earlier workers (Chaikoff and Connor, 1940 ; Chaikoff, Eichorn, Connor and Entenmann, 1943) showing that, with the lapse of time, the degree of fatty infiltration diminishes, even though the consumption of fat continues unabated. A further point, in this connection, demonstrated by these present results, is that the heavier this infiltration in the early stages of the experiment, the more rapid and marked its subsequent decline.

The severity and speed of development of diffuse, hepatic, fibrosis is proportional to the preceding fatty infiltration (Fig. 1). Thus on diet F.4 per cent only a mild fibrosis was present at 100 days, while on diet F.50 per cent, at the same time, the fully developed lesion had appeared. Further on diets F.4 per cent, F.16 per cent and F.30 per cent, animals survived in fair health for the full experimental period of 200 days ; on diet F.50 per cent all died or were ill with the lesion before 150 days.

Further evidence relating fatty infiltration to diffuse fibrosis is provided by the observation that fatty infiltration appears first and is heaviest in the left lobes. This is to be attributed to the right lobes receiving mainly blood from the small intestine, carrying the products of digestion, while the left receive blood mainly from the colon and spleen (Séregé, 1902 ; Copher and Dick, 1928 ; Glynn and Himsworth, 1944 ; Himsworth and Glynn, 1944). As a consequence the right lobes receive, and apparently abstract, the small amounts of lipotropic factors available on these diets and so fatty infiltration in them is under some

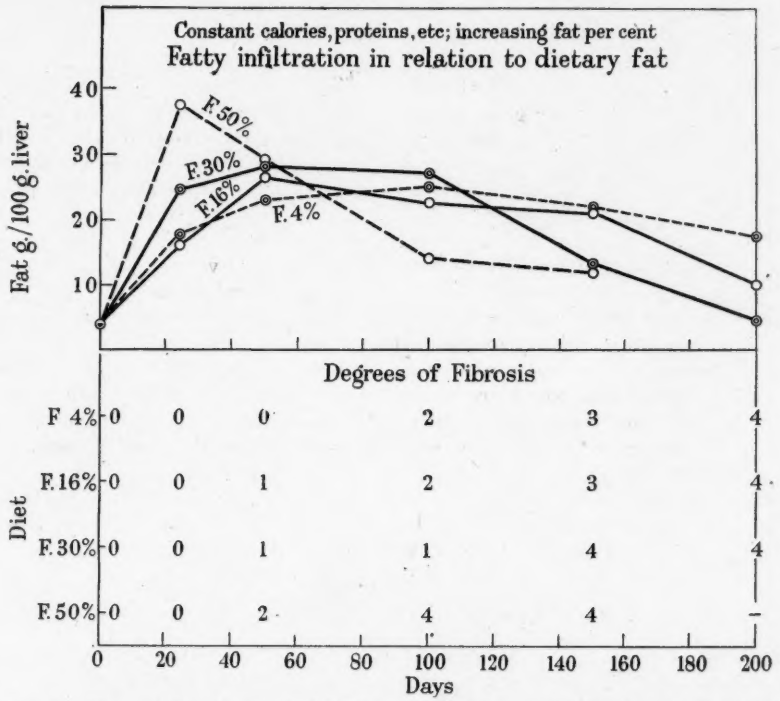

FIG. 1.—Showing that the speed of development and degree of diffuse hepatic fibrosis is proportional to the degree and duration of the preceding fatty infiltration.

restraint. As diffuse fibrosis develops throughout the liver the lesion in the left lobes is always a grade more advanced than in the right.

Effect of Lipotropic Factors.

Fig. 2 shows the effect of supplements of choline on rats which were all taking diet F.50 per cent. It will be seen that 5 mg. choline/rat/day reduces markedly the degree of fatty infiltration while 20 mg. choline/rat/day restrain the fat content of the liver within normal limits. These effects are reflected in the development of diffuse fibrosis. On diet F.50 per cent alone conspicuous lesions develop early ; on diet F.50 Ch.5 only the earliest trace of damage can be found at 150 to 200 days ; on diet F.50 Ch.20 no fibrosis whatever appeared. This preventive action of choline can only be attributed to the action of choline on fatty infiltration for it has been shown that choline affords no protection against the development of dietetic necrosis (Himsworth and Glynn, 1944).

In another series of experiments in which the same six diets were used, a small supplement of liver extract was given. This proved to have a significant lipotropic action and, throughout the experiment, restrained the liver fat content to 15 per cent or less. This experiment continued for 450 days. No fibrosis

developed on the F.4 per cent, F.16 per cent, F.30 per cent, F.50 per cent Ch.5 or F.50 Ch.20 diets. On the F.50 per cent diet traces were found after 400 days.

These results show unequivocally that the amino-acid content of the diets used was adequate to prevent massive necrosis and further that, on such diets,

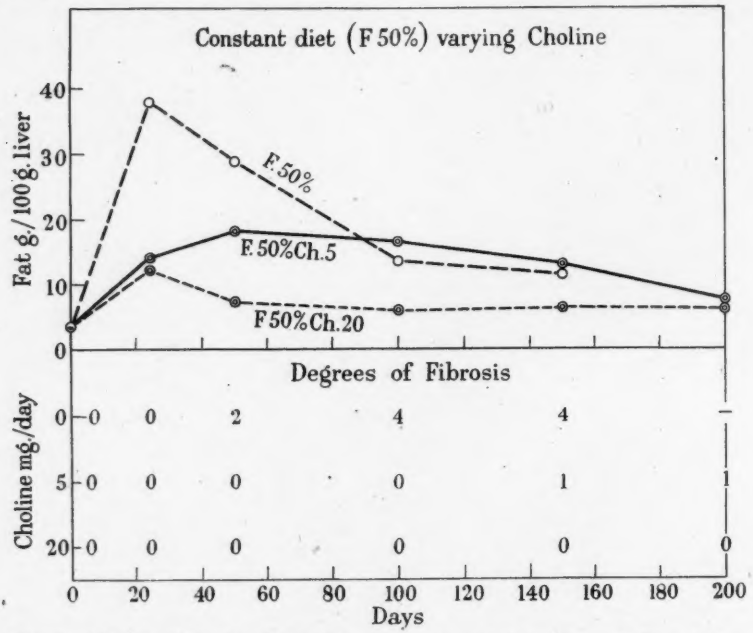

FIG. 2.—Showing that when fatty infiltration is prevented, by giving choline, diffuse hepatic fibrosis is also prevented.

diffuse hepatic fibrosis does not develop unless fatty infiltration of adequate severity is produced and maintained for the requisite length of time. The degree of fatty infiltration required and the time factor are within limits related variables. With liver fat contents of less than 15 per cent diffuse fibrosis does not seem to develop at all. At higher levels of fatty infiltration its speed of development is clearly related to the degree of such infiltration.

DISCUSSION.

It is evident from the preceding results that an insidious diffuse fibrosis, without any antecedent necrosis but correlated with the degree and duration of a preceding fatty infiltration, develops at levels of protein intake which are adequate to protect against dietetic massive necrosis. In dietetic experiments this particular anatomical form of fibrosis only develops on the basis of heavy fatty infiltration; fatty infiltration is, therefore, at least, its approximate cause. That, in these experiments, it is the actual infiltration with fat, and not some

injurious component of dietary fats, which causes the injury is shown by the observation that when fatty infiltration is prevented by choline no lesion occurs, and that diffuse fibrosis develops whether the fat in the liver is derived from exogenous sources (diet F.50 per cent) or from endogenous sources (diet F.4 per cent), as occurs when lipotropic factors are deficient (Barrett, Best and Ridout, 1938). Looking farther afield, however, it is evident that a morphologically identical fibrosis develops in the presence of hepatic infiltratiom with substances other than fat. Experimentally it can be produced by giving cholesterol to rabbits (Chalatow, 1914 ; Himsworth, 1938 ; Leary, 1941) and by intravenous injection of inutilizable macromolecular substances such as the polyvinyl alcohols (Hueper, 1944). Perhaps the diffuse fibrosis following injection of silica gels (Gye and Purdy, 1922) is in the same class. In man a similar fibrosis occurs after fatty infiltration (Connor, 1937, 1938), in Gaucher's disease (Himsworth, 1947), in xanthomatosis (Thannhauser, 1940) and sometimes in the survivors of glycogen disease (van Creveld, 1939 ; Lindsay, Ross and Wigglesworth, 1935). In all these cases there is no question of inadequate dietary protein and the conclusion seems inescapable that *infiltration of the liver as such, and irrespective of the nature of the infiltrating agent, leads ultimately to the development of a diffuse hepatic fibrosis.*

But a diffuse hepatic fibrosis, morphologically indistinguishable from that produced by infiltration, can develop after repeated, frequent, attacks of centrilobular necrosis. Such can be produced experimentally in rats by frequent injections of carbon tetrachloride (Lamson and Wing, 1926*a*, *b*, *c* ; Bollman and Mann, 1931 ; Cameron and Karunaratne, 1936). This fibrosis was originally thought to centre on the portal tracts. Undoubtedly the portal tracts are heavily involved in the established lesion, but Ashburn, Endicott, Daft and Lillie (1947) have shown, and we have confirmed, that the earliest fibrosis often develops round the central veins and that the subsequent evolution of the lesion follows similar lines to that of post-infiltrative fibrosis. It seems to us that this toxipathic lesion may provide the clue to understanding the mechanism by which infiltration leads to fibrosis. Connor (1937, 1938) originally suggested that such infiltration might interfere with the intralobular circulation and so impair the nutrition of the parenchyma. We have now considerable evidence that this suggestion is essentially correct.

It has been shown (Himsworth, 1947 ; Glynn and Himsworth, 1947) that following a subcutaneous injection of carbon tetrachloride the parenchymal cells swell, compress the sinusoids and so retard the intralobular circulation. Centrilobular necrosis, however, does not appear until 8 to 10 hours after the injection, that is some 6 hours after the intralobular circulation has been impeded. It takes 6 hours for the effects of completely occluding the hepatic circulation to become manifest as the alteration in cellular structure we recognize as necrosis. We have, therefore, suggested that the centrilobular necrosis following carbon tetrachloride is the expression, not of a direct action of the poison of the central parenchyma, but of relative ischaemia of the central zones consequent upon a retardation of the blood flow in the intralobular sinusoids so that the blood reaching the centre is depleted of oxygen and nutriments. A similar but less severe restriction of the intralobular circulation can be demonstrated in fatty infiltration. If rats are given the F.50 per cent diet then, after 4 or 5 days,

a patchy fatty infiltration of the liver will have developed. Following the technique used in the previous experiments indian ink is injected into the spleen of the living anaesthetized animal. From here it is carried by the circulating blood through the portal venous system to the liver. In this way it can be demonstrated that the patency of the intralobular sinusoids varies inversely with the degree of fatty infiltration. Where infiltration is absent the sinusoids are shown as wide channels; where infiltration is confined to the central zones as wide channels in the periphery but thin streaks among the fat laden cells in the centre; and where infiltration is heavy the sinusoids can be distinguished only with difficulty. It is evident that fatty infiltration offers a definite impediment to the intralobular circulation. But this would not appear to be so great as in carbon tetrachloride poisoning. Deprivation of the portal blood supply leads to atrophy of the hepatic parenchyma (Solowieff, 1875; Rous and Larimore, 1920) while deprivation of oxygen causes rapid and frank necrosis. Severe retardation of the intralobular circulation, as in carbon tetrachloride poisoning, might be expected to cause anoxia of the central cells and subsequent necrosis; milder retardation, as is observed in fatty infiltration, to unobtrusive atrophy of the central cells. Presumably parenchymal atrophy, after ligation of the portal vein, results from deprivation of a substance normally carried in the portal venous blood. It is probable that such might be a nutriment produced by digestion in the small intestine. It is, therefore, suggested that infiltration—fatty or otherwise—produces its ill effects by retarding the intralobular circulation so that the slowly percolating blood in the sinusoids is largely depleted of some nutriment, essential to the hepatic parenchyma, by the time it reaches the centre of the lobule. Atrophy of the central cells follows, the reticulin round the central vein collapses and so the process, which is observed in chronic fatty infiltration and repeated poisoning with carbon tetrachloride, is set in train; and ultimately a diffuse hepatic fibrosis develops. In the last analysis this sequence is due to a deficient supply of some as yet unrecognized nutriment required by the hepatic parenchyma, but it is evident that this deficiency would not have occurred in the absence of heavy prolonged infiltration. Chronic infiltration is, therefore, the essential, if not the ultimate cause, of dietetic diffuse hepatic fibrosis.

SUMMARY.

1. Groups of rats were given a series of diets so constructed that equicaloric amounts provided the same intake of protein, vitamins and salt, but increasing amounts of fat. The protein, and amino-acid and vitamin supplements were adequate to protect against the development of dietetic massive necrosis.

2. Fatty infiltration of the liver developed in the different groups at a speed and to a degree proportional to the amount of fat in the respective diets. Following such fatty infiltrations, and without any antecedent necrosis, a diffuse hepatic fibrosis, indistinguishable from classical portal cirrhosis, developed insidiously. The speed of development and degree of this fibrosis were proportional to the degree of the preceding fatty infiltration.

3. When fatty infiltration was prevented by supplements of choline the livers remained entrely normal, although in animals on the same diet but without choline, marked fibrosis developed.

4. A similar diffuse hepatic fibrosis develops after other infiltrations, such as polyvinyl alcohols, cholesterol, kerasin, etc. The development of diffuse hepatic fibrosis is, therefore, irrespective of the nature of the infiltrating agent.

5. Infiltration impedes the intralobular circulation. It is believed that fatty infiltration produces its injurious effects by thus restricting the circulation, and so causing malnutrition and atrophy of the lobular parenchyma which leads on to the development of a diffuse fibrosis.

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THE SURVIVAL OF THE VIRUS OF FOOT-AND-MOUTH DISEASE IN BLOOD AT 37°C.

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DURING experiments on the vaccination of guinea-pigs against foot-and-mouth disease, several batches of infective guinea-pig blood, with crystal violet in a concentration of 0·05 per cent as a bacteriostatic agent, were incubated at 37° C. to inactivate the virus. It was at once noticed that the time necessary for inactivation was greater with citrated than with defibrinated blood. As the factors involved in such discrepancies would deserve consideration in the development of the technique of preparation of vaccines from blood, some investigation seemed desirable of the conditions influencing the duration of virus survival in blood at 37° C.

METHODS.

Strains of virus.

As these observations were confined to guinea-pigs, the three stock strains which have been maintained by serial passage in guinea-pigs were used.

They are as follows :

Strain 1 (Vallée " 0 " type) isolated from an outbreak in cattle in Great Britain in March, 1924.

Strain GB (Vallée " A " type) a guinea-pig adapted strain received from the Island of Riems in 1929.

Strain GC (Waldmann " C " type) a guinea-pig adapted strain received from the Island of Riems in 1933.

Between serial passages in guinea-pigs, these strains are stored as vesicle fluid, collected in M/25 phosphate buffer, pH 7·6, filtered through a Seitz E.K. pad and then diluted 1/50 in a mixture of equal parts of the phosphate buffer and glycerine. Such preparations are commonly infective at dilutions of 10^{-5} or 10^{-6}, and produce severe vesicular lesions at the site of inoculation on the plantar pads of guinea-pigs within 24 hours of inoculation. Blood collected from infected guinea-pigs at the height of the disease is generally infective when diluted to 10^{-3} or 10^{-4}.

Guinea-pigs.

The animals used were all from the breeding colony established to meet the needs of the Institute. Healthy animals of 400 g. or over were used. On occasion, guinea-pigs which had failed to react to a test inoculation, were infected

later for use as donors of infective blood. Blood from such donors was satisfactory in respect of virus content, and appeared to behave as blood from fresh animals. All guinea-pigs under experiment were housed in galvanized buckets with sawdust as litter. During the 7 or 8 days of examination after a test inoculation, the buckets were not cleaned out, and the only handling of the animals was for examination. The food consisted of a morning feed of oats and bran, and an afternoon feed of greenstuff or roots.

Collection and routine treatment of infective blood.

Blood was collected by cardiac puncture from the infected guinea-pigs. If anticoagulants were used, they were contained in solution in the syringe into which the blood was aspirated. For defibrination, blood was drawn into a clean syringe and then expelled into a sterile, glass bottle, and the fibrin removed by shaking with glass beads. In all cases, a tube of nutrient broth, and an agar slope, were inoculated with the blood sample, and, if a growth of micro-organisms resulted, the blood sample was not used.

At this stage, a sample of each batch of blood (the batch usually consisting of blood from five or more donor guinea-pigs) was taken for titration of the virus content. A 1 per cent solution of crystal violet DuPont, " extra pure," was then added to the blood so that the final concentration of the dye was 0·05 per cent. Incubation of the blood plus crystal violet was carried out in rubber-stoppered bottles in an incubator at 37° C.

Titration of the virus content of infective bloods.

Throughout this work, the estimation of virus titre was carried out by making serial decimal dilutions of blood or virus suspension, in phosphate buffer at pH 7·6. Each dilution of a chosen range was inoculated intradermally into the plantar pads of three guinea-pigs, within one hour of the preparation of the dilution. The virus content of the infective blood was then expressed in one of two ways—either :

(1) As the highest dilution at which a stated proportion of inoculated guinea-pigs developed lesions (e.g. 10^{-4}, 2/3 indicating that 2 out of 3 guinea-pigs inoculated with a 10^{-4} dilution developed lesions) ; or—

(2) As the two dilutions concerned when 2 out of 3 guinea-pigs reacted in one group and 1 out of 3 in the group receiving the next higher dilution (e.g. $10^{-3}/10^{-4}$ indicating that 2 of 3 guinea-pigs inoculated with a 10^{-3} dilution and 1 of 3 inoculated with a 10^{-4} dilution developed lesions). Such an estimate was probably sufficiently precise for the purpose of these experiments since other factors affecting the survival of virus appeared to be of greater significance than the original titre. The expression of titre as the 50 per cent end-point would not be justified unless observations had been made on larger numbers of guinea-pigs, and the nature of the experiments would not warrant this refinement.

Estimation of the time necessary for inactivation.

Samples were withdrawn at 24-hour intervals from all batches undergoing test, and inoculated into groups of three guinea-pigs. As in the case of the titration, calculation of the time taken for the infectivity of the material to

decrease to any arbitrary level, such as the 50 per cent positive end-point, was not used. The value obtained from the number of guinea-pigs used would be no more precise an estimate of viability than the statement of the last day on which virus could be detected by the development of lesions in the guinea-pigs concerned. Hence all values for the viability of virus in particular substrates are, in fact, the numbers of the days from the beginning of incubation on which virus was last detected.

RESULTS

1. *The effect of decalcifying anticoagulants.*

The result of a typical experiment is shown in Table I.

The viability of virus in citrated blood was, in this case, more than twice as long as in defibrinated blood, though, on the evidence of the titration, more virus was present initially in the latter. Addition of citrate to the defibrinated blood prolonged the survival of the virus, although the end-point was not precisely determined. Similar observations have been made in many experiments, and are represented graphically in Fig. 1, which shows the survival times for 8

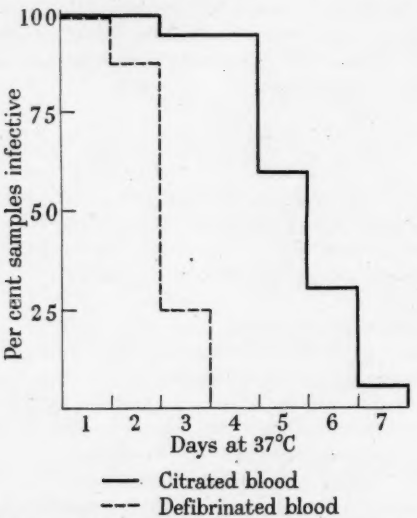

FIG. 1.—The survival of the virus of foot-and-mouth disease in blood at 37° C.

samples of defibrinated and 20 samples of citrated blood. Not all these results were obtained with the same strain of virus, and the initial titre of the infective bloods also varied from experiment to experiment with both defibrinated and citrated samples. In spite of these factors it is clear that a marked difference in survival time exists between the two series. The mean viability in citrated blood is 4·9 ($\sigma = 1{\cdot}18$) and in defibrinated 2·1 ($\sigma = 0{\cdot}6$). The difference is therefore statistically significant ($t = 13{\cdot}5$ at 26 degrees of freedom).

Other experiments have shown that the effect of citrate on the survival of virus in blood is related to its action on Ca ions and not to the other differences which exist between citrated and defibrinated blood. The behaviour of the virus in defibrinated blood with citrate added, as recorded in Table I, is almost the same as in citrated blood. In citrated blood from which the leucocytes had been removed by filtration through cotton wool, survival for 4 days was recorded, compared with 5 days in unfiltered blood and 2 days in defibrinated blood, from the same group of animals. Furthermore, virus has been shown to survive better in plasma than in serum. In a direct comparison, virus in a sample of citrated plasma was not inactivated under 3 days of incubation, while the serum from defibrinated blood was no longer infective at 1 day. On other occasions, samples of citrated plasma have been shown to be infective after 4 days at 37° C., at which time virus has not been demonstrated in defibrinated blood from guinea-pigs.

The other " decalcifying " anticoagulants appear to have an effect similar to citrate. An experiment in which three batches of blood, from the same group of guinea-pigs, were prevented from clotting respectively by sodium citrate, sodium fluoride and potassium oxalate gave the result shown in Table II.

On the other hand, virus in heparinized and in defibrinated blood behaves similarly. This is shown in the experiment summarized in Table III.

It is clear from these experiments that the presence of calcium ions in guinea-pig blood or serum hastens the inactivation of the virus of foot-and-mouth disease in these media at 37° C.

2. *The influence of the initial virus titre.*

Although no trend was observed in the direction of a higher initial titre of citrated blood samples as compared with defibrinated, it was thought desirable to investigate the correlation of initial titre with survival of virus at 37° C. First, from the observations already described, six comparable samples of citrated blood were selected, differing only in respect of initial titre. The observations on titre and survival of virus gave the result shown in Table IV.

TABLE IV.—*Comparison of Titre of Citrated Infective Bloods with Observed Survival of Virus.*

Initial titre.	Observed period of survival in days.
10^{-3}	5
10^{-3} to 10^{-4}	4, 5, 6
10^{-4}	5, 6

From this, it appeared that no positive correlation existed within the ten-fold range of titre observed. Further corroboration was then sought in experiments in which artificial variations in virus titre were produced by addition of infective blood or vesicle fluid to normal blood. Since the range of titres in the experiments described in section 1 was approximately 10-fold, the experiment using mixtures of infective and normal blood was arranged so that the proportions were 1 : 30, 1 : 10, 1 : 3 and 1 : 0. The initial titre of the infective blood used was 10^{-3} to 10^{-4} and the viability of the virus in the four mixtures is shown in Table V. Both infective and normal bloods were citrated at the time of collection.

TABLE I.—*Influence of Citrate on Survival of Virus.*

	Initial titre.	Days of incubation. 2.	3.	4.	5.	6.	7.
Citrated (0·4 per cent citrate)	10^{-3} (2/3)	++ ++ ++	++ ++ ++	++ ++ ++	++ 00 00	00 00 00	00 00 00
Defibrinated	10^{-4} (2/3)	++ ++ ++	00 00 00	00 00 00	- - -	- - -	- - -
Defibrinated with 0·4 per cent citrate added	10^{-4} (2/3)	++ ++ ++	++ ++ 00	++ ++ 00	- - -	00 00 00	00 00 00

Virus Strain GB. Blood from 20 donor guinea-pigs, taken 40 hours after inoculation with stock virus, when all showed full primary lesions, and 12 rising secondary lesions.

N.B.—++ indicates guinea-pig developed primary and secondary lesions; 00 indicates guinea-pig remained healthy; - - - not tested.

TABLE II.—*Effect of Decalcifying Anticoagulants.*

Anticoagulant.	Days of incubation. 2.	3.	4.	5.	7.
Sodium citrate 0·4 per cent	- - -	++ ++ ++	++ 00 00	++ ++ +0	00 00 00
Sodium fluoride 0·3 per cent	- - -	++ ++ ++	++ ++ ++	++ ++ ++	00 00 00
Potassium oxalate 0·1 per cent	++ ++ ++	++ ++ ++	++ ++ ++	++ 00 00	00 00 00

N.B.—++ indicates guinea-pig developed primary and secondary lesions; +0 indicates guinea-pig developed primary lesions only; 00 indicates guinea-pig remained healthy; - - - not tested.

TABLE III.—*Effect of Heparin.*

	Days of incubation. 2.	3.	4.	5.	6.	7.
Citrated	++ ++ ++	++ ++ ++	++ ++ ++	++ ++ +0	++ ++ ++	00 00 00
Heparinized	++ ++ ++	00 00 00	00 00 00	- - -	- - -	- - -
Defibrinated	++ ++ ++	++ ++ ++	00 00 00	- - -	- - -	- - -

N.B.—Symbols as Table II.

TABLE V.—*Effect on Survival of Virus of Artificial Adjustment of Titre by Mixing of Infective and Normal Bloods.*

Proportion of infective to normal blood.	Days of incubation. 2.	3.	4.	5.	6.	7.	8.
1 : 30	++ ++ 00	++ ++ 00	++ 00 00	00 00 00	00 00 00	- - -	- - -
1 : 10	++ ++ ++	++ ++ 00	++ ++ 00	00 00 00	00 00 00	- - -	- - -
1 : 3	++ ++ ++	++ ++ ++	++ ++ ++	00 00 00	00 00 00	00 00 00	- - -
1 : 0	- - -	++ ++ ++	++ ++ 00	++ 00 00	00 00 00	++ 00 00	00 00 00

N.B.—Symbols as Table I.

TABLE VI.—*Effect on Survival of Virus of Artificial Adjustment of Titre by Addition of Virus to Normal Blood.*

	Proportion of vesicle fluid to normal blood.	Estimated titre.	Days of Incubation. 2.	3.	4.	5.	6.	7.
Citrated.	1 : 100	$10^{-4}/10^{-5}$	- - -	++ ++ ++	++ ++ ++	++ ++ ++	++ ++ 00	00 00 00
Citrated.	1 : 1000	$10^{-3}/10^{-4}$	- - -	++ ++ ++	++ ++ ++	++ 00 00	00 00 00	00 00 00
Citrated.	1 : 10,000	$10^{-2}/10^{-3}$	++ ++ ++	++ ++ ++	00 00 00	00 00 00	00 00 00	- - -
Citrated.	1 : 100,000	$10^{-1}/10^{-2}$	++ 00 00	00 00 00	00 00 00	- - -	- - -	- - -
Defibrinated.	1 : 1000	$10^{-3}/10^{-4}$	++ ++ ++	++ ++ +0	00 00 00	00 00 00	- - -	- - -

N.B.—Symbols as Table II.

In this experiment there is a slight tendency for the survival time to decrease with the decrease in the virus present in the initial mixture. It is obvious, however, that the effect of a ten-fold difference in titre is much less than the effect of the addition of citrate to defibrinated samples.

When filtered vesicle fluid was added to normal blood in a further experiment, the opportunity was taken to make the range of titre much wider. Serial ten-fold dilutions of filtered vesicle fluid diluted 1/10 in phosphate buffer were prepared in normal citrated blood, so that the titres of successive dilutions, estimated by preliminary titration of the filtrate, were $10^{-4}/10^{-5}$, $10^{-3}/10^{-4}$, $10^{-2}/10^{-3}$ and $10^{-1}/10^{-2}$. At the same time, a dilution of vesicle fluid in defibrinated normal blood was prepared, calculated to have a titre of $10^{-3}/10^{-4}$. The reactions of the guinea-pigs inoculated with samples taken at daily intervals during incubation are shown in Table VI.

In this instance, the fall in survival time in successive ten-fold decrements is greater than would be expected from the experiment using infective blood, or from the results reported in section 1. It is possible that the use of vesicle fluid introduces another factor, and the result may hence differ from that obtained with virulent blood where the virus might be described as "indigenous." The survival of added virus in defibrinated blood is approximately the same as in citrated blood of ten-fold lower titre, and therefore is considerably less than in citrated blood of the same titre.

3. *The effect of the blood corpuscles.*

Observations were made on the survival of virus at 37° C. in plasma and serum. It was at once apparent that separation of the corpuscles from an infective blood led to a great decrease in the duration of survival. Figures obtained on whole blood and the serum and plasma prepared from it are given in Table VII.

TABLE VII.—*Comparison of Survival of Virus in the Presence and Absence of the Blood Corpuscles.*

Blood.	Whole blood.	Serum or plasma.
Citrated	6	Less than 2
,,	5	2
,,	6	4
,,	4	Less than 3
Defibrinated	3	,, 1

N.B.—The figure given is the last day on which virus could be demonstrated in the particular sample.

To some extent, the shorter survival time in serum or plasma might be attributed to the diminution in titre which may occur with the separation of corpuscles from virulent blood. In one of the experiments cited in Table VII, however, where there was no fall in titre, the survival in plasma was 4 days, as compared with 6 days in the citrated whole blood.

Another possible explanation may lie in the influence of the oxidation-reduction potential of the medium on virus survival. The experiments of Zinsser and Tang (1929) and Perdrau (1931) with herpes, Mueller (1928) with Rous sarcoma,

Edwards (1931) with foot-and-mouth disease, Bang and Herriott (1943) with equine encephalomyelitis, and others, all suggest a "reducing" medium as favouring virus survival. The answer to the present problem was therefore sought in attempts to replace the separated cells by "normal" cells from a non-infected animal, or by the addition of reducing agents such as cysteine hydrochloride.

The results of these experiments are given in abbreviated form in Table VIII.

TABLE VIII.—*Attempts to Replace the Indigenous Cells of Infective Blood.*

	Days of incubation.						
	1.	2.	3.	4.	5.	6.	7.
Citrated blood	—	—	+	+	+	+	0
Citrated plasma	+	+	+	+	0	0	0
Citrated plasma + 1/1000 cysteine	+	+	+	+	0	0	0
Citrated plasma + Normal cells	+	+	+	+	+	0	0
Citrated blood + 1/1000 cysteine *in vacuo*	+	+	+	+	+	0	0

N.B.—In Table VIII + signifies a lesion produced in any one of the three guinea-pigs inoculated with each sample; 0 = no lesions; and — = sample not tested.

Partial success only attended the efforts to replace the indigenous cells of infective blood. It appears probable that the effect of removal of cells is due to factors of which the oxidation-reduction potential is only one.

4. *The influence of time of collection of blood in relation to the development of lesions.*

With the guinea-pig adapted strains of virus used, primary lesions were frequently observed at 16–18 hours after inoculation, and the early signs of secondary lesions, such as redness and swelling of the pads of the fore feet, at 36 hours. It has already been demonstrated by earlier workers (Galloway and Nicolau, 1928; Galloway, 1931) that virus is present in the circulating blood from the time of appearance of the primary lesions until 2 or 3 days later. It seemed desirable to obtain more precise information on the titre of virus in the blood during the early states of the disease, as a preliminary to investigating survival at 37° C. An experiment was made in which blood was collected into sodium citrate at intervals of six hours, from successive batches of five guinea-pigs of a group of 30, all inoculated with the same preparation of stock vesicle fluid. The pooled batches from each group were then titrated in the way described earlier, with the result shown in Table IX. The titres recorded in Table IX suggest that any difference in the survival of virus in incubated bloods collected during the period 24–48 hours after inoculation could not be attributed to variations in titre. That such differences do occur is indicated by the results of the following experiments.

TABLE IX.—*Titre of Virus in Infective Blood Collected at Various Stages in Development of Disease.*

Time from inoculation to collection of blood (hours).	Titre of pooled blood from five guinea-pigs.
18	$10^{-2}/10^{-3}$
24	10^{-3}
30	$10^{-3}/10^{-4}$
36	10^{-3}
42	$10^{-3}/10^{-4}$
48	$10^{-3}/10^{-4}$

In the first of these experiments the survival of virus was compared in two citrated samples taken at the 28th and 48th hours after inoculation. Virus of strain GC was used, and the titres of the blood samples were somewhat lower than those usually found in experiments with strains 1 and GB. The 28-hour sample had a titre of 10^{-2} to 10^{-3} and was non-infective after 5 days of incubation. The 48-hour sample had a titre of 10^{-2} and was non-infective at the first test at 3 days. Virus of strain GB was used in the second experiment. In this instance, the titre of the virus in both the 28- and 48-hour samples was 10^{-3}. Active virus was found in the 28-hour sample after six days at 37° C. while the 48-hour sample was no longer infective when first tested at 3 days.

A third experiment, with three citrated samples, taken at 24, 36 and 48 hours after inoculation is summarized in Table X.

TABLE X.—*Relationship Beteeen Time of Collection of Infective Blood and Survival of Virus.*

Time of collection of blood (hours after inoculation).	Titre.	Days of incubation.						
		1.	2.	3.	4.	5.	6.	7.
24	10^{-4}	—	+	+	0	+	0	—
36	10^{-4} to 10^{-5}	—	—	+	+	+	+	+
48	10^{-3} to 10^{-4}	+	+	+	+	0	0	—

N.B.—+ signifies a lesion produced in any one of the three guinea-pigs inoculated with each sample; 0 = no lesions; and — = sample not tested.

Here a tendency was exhibited towards a higher titre in the 36-hour sample, and this would probably accentuate the result. Nevertheless, there is again better survival of virus in blood collected at the time when the secondary lesions are not yet fully developed. It must be emphasized that these results were obtained with citrated blood. Defibrinated samples collected from groups similar to those shown in the table were tested for survival of virus at 37° C. In each case, no active virus could be detected after 3 days or more. The enhancing effect of the decalcifying anticoagulant is therefore necessary for the demonstration of the phenomenon.

DISCUSSION.

Until further information becomes available discussion of the probable mechanisms involved in the " citrate effect " is bound to be speculative. Further

investigations would involve a detailed study of the effect of the different constituents of the blood on the virus ; for example, the effect of the Ca ion may be direct on the virus, or it may be on some enzyme system which, operating in the blood, leads to destruction of the virus. Experiments on the effect of Ca ions on purified virus suspensions have not yet been carried out.

In all observations recorded, crystal violet in a concentration of 0·05 per cent had been added to the various media. The presence of the dye while it might complicate the explanation of the mechanisms involved in the inactivation of virus in the blood, does not seem likely to be an essential factor in the difference which is associated with the presence or absence of the Ca ion.

The effect of the time of collection is possibly due to some inherent property of the virus and its relation to the host. The characteristics of the virus entering the blood stream may alter with the progress of the lesion in which it is multiplying. Alternatively, an antibody response of the part of the host, too small to be measured in the usual way, but still affecting the survival of the virus over the prolonged period at 37° C., may be involved.

The experiments recorded were, however, undertaken to ascertain the main factors influencing the survival of virus in infective blood. The " citrate effect " could lead to serious trouble in the production of an inactivated blood vaccine if the technique were changed from the use of defibrinated to citrated or oxalated blood, without investigation of the infectivity of the product. If defibrinated blood is used, the other factors are of minor importance, since, in the experiments described, their demonstration was possible only in " decalcified " blood.

SUMMARY.

(1) The mean survival time at 37° C. of the virus of foot-and-mouth disease in infective guinea-pig blood is 2·1 days ($\sigma = 0·6$) if the blood be defibrinated and 4·9 days ($\sigma = 1·18$) if the blood be citrated.

This effect is correlated with the presence of Ca ions in defibrinated blood.

(2) The higher the initial titre of a sample of infective blood, the greater the time of survival of the virus.

(3) Removal of the corpuscles decreases the time of survival of virus.

(4) The survival time of virus varies with the time of collection of the infective blood in relation to the development of lesions in the donor guinea-pigs.

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THE ENHANCEMENT OF BACTERIAL INFECTIONS BY ADRENALINE

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FOLLOWING the observation that an intramuscular injection of adrenaline in a man had apparently induced a *Cl. welchii* gas gangrene at the injection site, Cooper (1946) demonstrated a marked decrease in the minimal lethal dose of *Cl. welchii*, when washed bacilli were injected into the muscle of guinea-pigs together with adrenaline. A 10,000-fold decrease occurred with 62·5 μg. adrenaline in a volume of 1 ml. Adrenaline thus enhances the infectivity of *Cl. welchii* as effectively as 2·5 per cent $CaCl_2$, but does so without the gross damage to the tissues produced by $CaCl_2$ in the concentrations commonly used. We have investigated the nature of this enhancement in experimental infections both by clostridia, and by a number of pathogenic aerobic bacteria. For many of the bacterial species tested, adrenaline decreased the number of living organisms required either to kill the animal, to establish a generalized infection, or to produce infective lesions of a given degree of severity. We have surveyed the infections following the injection of washed living bacteria into the skin of rabbits and guinea-pigs, measuring the size and severity of the local lesion, and into the thigh muscle of guinea-pigs, in this case determining the minimal lethal dose of the bacteria.

The enhancement of infections by adrenaline.

Bacteria in the skin of guinea-pigs and rabbits.—For intradermal tests, ten-fold dilutions of a suspension of washed bacteria, usually from an overnight growth in an optimal medium, were injected in 0·1 or 0·2 ml. quantities within 30 minutes of mixing with equal volumes of 0·85 per cent saline (the S suspension) or a solution of adrenaline in saline (the A suspension). At the same time a viable count was made of the suspension by the method of Miles and Misra (1938), except with suspensions of *Cl. oedematiens*, *Cl. septicum* and *Cl. tetani*, which were counted in nutrient agar shake cultures incubated anaërobically. The skin was depilated the previous night by a brief application of a barium sulphide powder to the clipped skin. When possible, both S and A injections were made into each of the animals, to permit comparisons unaffected by variations between animals. With ten-fold dilutions of culture, relatively large differences between the S and A lesions could be measured by the ratio of the minimal effective doses of the S and A suspensions.

Severity of the inflammatory skin lesion.—Precise comparison of lesions was often difficult, because the dose-response differed in the S and A series. Two titrations in Tables I and II illustrate the difficulties. The lesions are recorded as the areas of moderate, marked and gross inflammation, calculated from

$D^2\pi/4$, where D is the diameter of circular lesions, and $dD\pi/4$ where D and d are the major and minor axes of elliptical lesions. The area of marked inflammation includes that of the gross, and the area of moderate, that of marked and gross inflammation. Moderate inflammation is defined as moderate induration or oedema, and pink hyperaemic skin ; marked inflammation as pronounced induration or oedema, and deeply reddened skin ; and gross inflammation as very pronounced oedema or induration, and skin either purple with petechial haemorrhages, or yellowish-white as the result of suppuration or necrosis.

These responses are clearly not proportional to the dose. If we select " gross " inflammation as the threshold effect, the S:A ratio for *Ps. pyocyanea* (Table I)

TABLE I.—*The Severity of Inflammation Produced by Graded Doses of* Ps. pyocyanea, *in the Skin of a Guinea-pig, injected with* 2·0 μg. *Adrenaline* (*A*), *and without* (*S*).

Dose.	Day.	Area of inflammation (mm².). Moderate.*		Marked.		Gross.	
		S	A	S	A	S	A
16×10^7	1	800	490	706	415	227	227
16×10^6		382	188	314	154	64	64
16×10^5		78	223	50	176	12	78
16×10^4		3	195	0	78	0	6
16×10^3		3	16	0	4	0	0
16×10^7	2	572	314	490	113	314	113
16×10^6		154	165	113	132	0	50
16×10^5		28	143	20	113	0	13
16×10^4		3	71	0	50	0	0
16×10^3		3	4	0	0	0	0
16×10^2		0	3	0	0	0	0

* For the definition of the degrees of inflammation, see p. 21.

is about $16 \times 10^5 : 16 \times 10^4 = 10:1$ after one day, but 100:1 after two days. " Marked " inflammation gives 10:1 after both one and two days. " Moderate " inflammation is more equivocal ; the response to increasing doses of bacteria increases more gradually than with severer lesions. To minimize the effect of the varying dose-response in the S and A series, the S:A ratios were calculated from doses giving marked inflammation at the time of maximal development of the lesions (e.g. at one day for *Ps. pyocyanea*). When there was no marked inflammation in the S series, moderate inflammation was used as the indicating effect. Thus with *Bact. coli* (Table II) the 78 mm.² from 46×10^7 bacilli in the S series is equated with 124 mm.² from 46×10^4 in the A series.

In some cases there was no difference in the end-point in the two series, but for a given dose of bacteria the A lesion was larger. Some strains of *Staph. aureus*, which behaved in this way in rabbits, proved by finer titrations with two-fold dilutions to have S:A ratios ranging from 2:1 to 5:1.

The bacterial strains used were all smooth, and the exotoxin-producers among them were fully toxigenic, except when otherwise stated.

TABLE II.—*The Severity of Inflammation Produced by Graded Doses of* Bact. coli, *in the Skin of a Guinea-pig, Injected with* 2·0 μg. *Adrenaline* (*A*), *and without* (*S*).

Dose.	Area of inflammation (mm.²) after 1 day.					
	Moderate.		Marked.		Gross.	
	S	A	S	A	S	A
46×10^7	78	415	0	346	0	227
46×10^6	50	297	0	240	0	104
46×10^5	20	143	0	104	0	0
46×10^4	2	124	0	78	0	0
46×10^3	2	39	0	0	0	0
46×10^2	2	3	0	0	0	0

TABLE III.—*The Ratio of Minimal Infecting Doses of Washed Viable Bacteria Injected into the Skin of Guinea-pigs with* 2·0 μg. *Adrenaline* (*A*), *and without* (*S*).

	Indicating lesion,* and age in days.	Minimal effective dose of living bacteria.		Ratio.
		S	A	S:A
Cl. septicum (VS 54)	M, 1	$>7 \times 10^7$	7×10^2	$>10^5$:1
Cl. welchii (S.R. 9)	M, 1	6×10^6	6×10	10^5:1
Bact. coli	Mod, 1	46×10^7	46×10^3	10^4:1
Pr. vulgaris	M, 1	63×10^7	63×10^4	10^3:1
Str. pyogenes (Richards)	M, 1	8×10^6	8×10^3	10^3:1
Ps. pyocyanea	M, 1	16×10^5	16×10^3	10^2:1
Staph. aureus (Humphrey)†	M, 1	2×10^8	3×10^6	66 :1
Staph. aureus 21	G, 2	9×10^7	9×10^6	10 :1
Cl. oedematiens (Jolly)	M, 1	9×10^6	9×10^5	10 :1
Br. abortus	Mod, 2	16×10^7	16×10^6	10 :1
B. subtilis	Mod, 1	6×10^6	6×10^5	10 :1
Str. pneumoniae, I	M, 1	29×10^6	29×10^5	10 :1
Staph. aureus 15‡.	G, 2	34×10^6	34×10^6	1 :1
V. cholerae‡	Mod, 1	47×10^6	47×10^6	1 :1
Sh. sonnei‡	Mod, 1	11×10^7	11×10^7	1 :1
Sh. flexneri‡	M, 1	18×10^7	18×10^7	1 :1
Cl. histolyticum (CN 920)	M, 1	15×10^5	15×10^5	1 :1
H. pertussis†	M, 1	3×10^4	3×10^4	1 :1

* Mod., M and G = moderate, marked and gross inflammation (see p. 21).
† Tested in rabbit's skin.
‡ Measured as relative sizes of lesions from same dose, ratio A:S was about 5:1.

The noteworthy points about intradermal infections (Table III) are :

(*a*) the large minimal effective doses (m.e.d.) in terms of viable bacteria ;

(*b*) the wide range of S:A ratios ; and

(*c*) the haphazard distribution of bacterial species, when arranged in order of susceptibility to adrenaline enhancement.

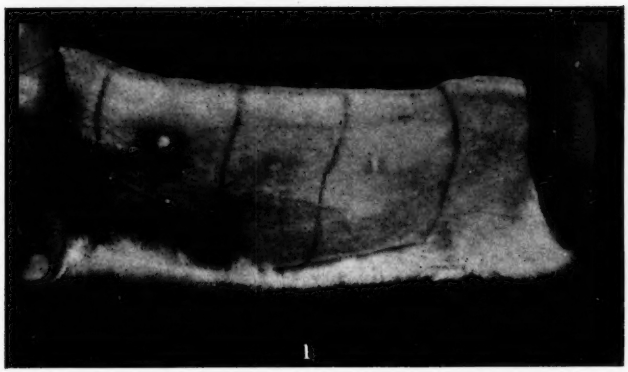

FIG. 1.—Left to right: washed organisms alone, 80, 40, 20 and 10 millions.

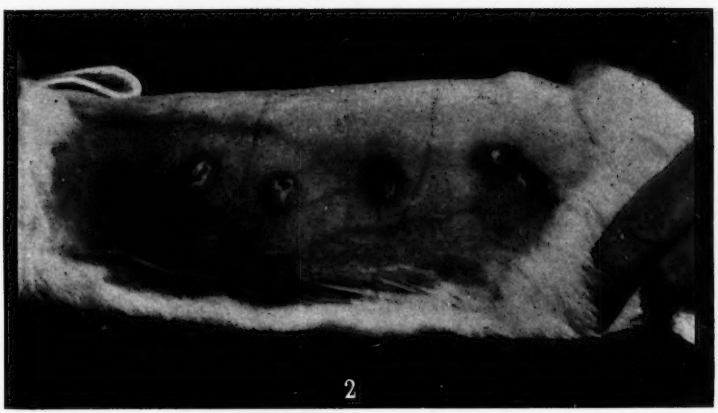

FIG. 2.—Left to right: washed organisms with 2·0 μg. adrenaline, 5, 10, 20, 40 and 80 millions.

FIG. 1 and 2.—Four day *Staph. aureus* infective lesions in the skin of a rabbit.

No safe general conclusion can be drawn about a given species from the results of testing one strain. In all, eight strains of *Staph. aureus* coagulase positive with human plasma were tested, and for these the S:A ratios varied from 5:1 to 100:1 (Figs. 1 and 2). Two strains of *Staph. aureus* from Professor Wilson Smith, both coagulase-positive with guinea-pig plasma, one (No. 15) α-toxigenic, the other (No 21) non-toxigenic, were tested several times in guinea-pigs; infections by No. 21 were consistently more enhanced than those by No. 15. Among the clostridia, the enhancement of *Cl. welchii* and *Cl. septicum* and the relative or total absence of it with *Cl. oedematiens* and *Cl. histolyticum* was striking. *Ps. pyocyanea* had an unexpectedly low m.e.d., and higher doses produced severe but non-ulcerating lesions that persisted, though slowly declining, for 14 days or more. For the bacteria marked ‡ in Table III the 1:1 ratios measured on a ten-fold scale are misleading; although the S and A end-points were the same, the A lesions were more severe than the corresponding S lesions, indicating a two- to five-fold enhancement of infections. Adrenaline had no effect on *H. pertussis* in the rabbit.

Bacteria in the muscle of guinea-pigs.—For the intramuscular tests the procedure was modified in that 0·5 ml. adrenaline solution was injected first, followed by 0·2 ml. of the graded doses of bacteria. Adrenaline enhanced intramuscular infections (Table IV) in the same degree as those in the skin, where the parallel test was made; in addition, *C. diphtheriae* infections were enhanced 100-fold, and *Cl. tetani* not at all.

TABLE IV.—*The Ratio of Minimal Lethal Doses of Washed Viable Toxigenic Bacteria by the Intramuscular Route in Guinea-pigs with 2 μg. of Adrenaline (A), and Without (S).*

Bacterium.	Minimal lethal dose of viable bacteria. S	Minimal lethal dose of viable bacteria. A	Ratio. S:A
Cl. septicum (VS 54)	$>7 \times 10^7$	7×10	$>10^6$:1
Cl. welchii (S.R. 9)	5×10^7	5×10^2	10^5:1
C. diphtheriae, gravis	15×10^4	15×10^2	10^2:1
Cl. oedematiens (Jolly)	9×10^5	9×10^4	10 :1
Cl. tetani (T 67)	1×10^3	1×10^3	1 :1
Cl. histolyticum (CN 920)	15×10^6	15×10^6	1 :1

Tests of other sites and organisms.—A few other tests were made. In the rabbit skin, 2 μg. adrenaline enhanced the lesions made by vaccinia virus ten-fold; the readings were made after 7 and 10 days. In the mouse there was no enhancement of the LD50 of a highly mouse-virulent *Str. pneumoniae* Type I, given either intraperitoneally or subcutaneously in the ear; 10–15 mice were used for each dose. And in the same animal, Dr. C. H. Andrewes reports that the infectivity of Type A influenza virus by nasal instillation 0·05 ml. virus supension was not altered by adding 100 μg. adrenaline to each ml. of suspending fluid.

The enhancement of intoxication by adrenaline.

The enhancement of infection by bacteria like *B. subtilis*, *Sh. sonnei*, and *Sh. flexneri*, none of which is particularly pathogenic for the guinea-pig, suggested

that the adrenaline might be enhancing intoxication, not infection. It is clear from Table V, however, that though clostridial exotoxins were enhanced two-

TABLE V.—*The Ratio of Minimal Effective Doses of Preparations of Bacterial Endo- and Exo-toxins in the Skin of Guinea-pigs and Rabbits Injected with* 2·0 μg. *Adrenaline* (*A*), *and Without* (*S*).

Toxin.	Animal.	Indicating reaction.	Ratio of m.e.d., S:A
Cl. septicum	Guinea-pig	Necrosis	4:1
Staph. aureus	Rabbit	,,	4:1
Cl. welchii	Guinea-pig	,,	2:1
Cl. oedematiens	,,	,,	2:1
Cl. histolyticum	,,	,,	2:1
C. diphtheriae	,,	Inflammation	1:1
Br. melitensis	,,	,,	1:1
Pr. vulgaris	,,	,,	1:1
H. pertussis	Rabbit	,,	1:1

to four-fold, *C. diphtheriae* toxin, the endotoxins of *Br. melitensis* (Miles and Pirie, 1939), *Pr. vulgaris* (a fraction corresponding to the F68 fraction of *Salm. typhi-murium* (Raistrick and Topley, 1934)), and *H. pertussis* toxin (Evans and Maitland, 1937) were unaffected. The enhancement of intoxication bears no constant relation to the enhancement of infections, for the S:A ratios for the toxins of *Cl. oedematiens* and *Cl. welchii* are of the same order, whereas the ratios for the infections are respectively 10:1 and 100,000:1. That intoxication, as distinct from invasion by multiplying bacteria, may contribute to the enhancement in infective A lesions was evident in *Cl. welchii* lesions (see p. 26). In the S lesions one hour old there was marked inflammation and leucocytosis, but little necrosis; in the A lesions no inflammation or leucocytosis, but incipient characteristic muscle necrosis.

On the other hand, such exotoxins as may be injected with the infecting dose of washed *Staph. aureus* were not demonstrably affected by adrenaline, as the following experiments show. Rabbits received 10,000 I.U. penicillin intravenously 3 hours before, and 2, 4 and 6 hours after the intradermal injection of graded amounts of washed, α-toxigenic *Staph. aureus* (Humphrey) mixed with 2·0 μg. adrenaline. Only the largest dose, 80×10^6 cocci, produced a moderate lesion 50 mm.^{2} in area, whereas in animals without penicillin, the same dose produced a necrotic lesion 445 mm.^{2} in area. A similar result followed the addition of 10 I.U. penicillin to the adrenaline-bacterium mixtures; thus in one rabbit 20×10^6 cocci with penicillin produced no lesion, and without penicillin a 980 mm.^{2} lesion. Clearly, the local intoxication was consequent on the multiplication of the staphylococci, and not due to preformed exotoxin whose action was enhanced by the adrenaline.

We may conclude that the greater part of the adrenaline effect is upon infection as distinct from intoxication by substances contained in the inoculum.

The Mode of Action of Adrenaline.

Speculation about the action of adrenaline in enhancing the infective process must clearly start with the acute and severe constriction of the arterioles that is produced by the relatively large doses used, resulting in a diminution of the blood flow through the capillary vessels distal to them. As a consequence, we may expect not only a diminution of the normal fluid and gaseous exchanges of the tissues, but also of many of the characteristic features of the inflammatory response to infective bacteria; and in particular exudation, and diapedesis of leucocytes, since the continuous supply of blood fluid and cells necessary for these phenomena is cut off.

The visible effects of 2·0 μg. adrenaline intradermally wear off in 1–2 hours, and, as far as the local resistance of the tissues is concerned, there is no after-effect. When graded doses of washed *Staph. aureus* or *Str. pneumoniae* were injected into normal guinea-pig skin and into the site of injection of 2·0 μg. adrenaline made 1 minute, 2 hours and 4 hours previously, the "1 minute" lesion was fully enhanced, the "2 hour" slightly, and "4 hour" lesion not at all. Here there was no gross after-effect of the adrenaline, its action being confined to the period of its manifest physiological activity. Moreover, with *Staph. aureus* in both rabbit and guinea-pig skin, the injection of 2·0 μg. adrenaline 2 and 4 hours after the injection of the bacteria did not enhance the infection, showing that with this organism at least, the infective process cannot be modified by adrenaline after a certain critical period.

As a working hypothesis, therefore, it may be assumed that during the temporary conditions in adrenaline-treated tissue certain bacteria are able to grow or adapt themselves to growth more readily than they do in normal tissue; and that the favourable consequence of adrenaline action is the relative absence of bactericidal substances and cells normally derived from the blood.

It is easy to establish that adrenaline inhibits both diapedesis of leucocytes and exudation.

Inhibition of exudation.

Depilated albino guinea-pigs weighing 250–350 g. were given 0·3–0·4 ml. of 5 per cent pontamine sky blue 6X (Gurr, London) intravenously, and injected intradermally with solutions of various substances known to increase capillary permeability, which was estimated by the marked staining of the injection site by exuded dye-stained blood fluids. Adrenaline, 3 3 μg. in 0·1 ml. injection fluid, inhibited the deep staining of tissues induced in 10–30 min. by 0·1 mg. histamine, by 1 mg. bacteriological peptone, and by maximal sub-necrotizing doses of the toxins of *Cl. welchii*, *Cl. septicum* and *H. pertussis*; and inhibited for 1–2 hours the lesser and more slowly developing stain induced by heavy suspensions of washed *Cl. welchii* and *Staph. aureus* containing about 10^{10} organisms per ml.

Inhibition of diapedesis.

Washed 18-hour living *Staph. aureus* and *Cl. welchii* and *B. subtilis* spores were suspended either in 0·85 per cent saline or 0·5 per cent peptone water, together with a trace of indian ink to mark the site of injection. These suspensions, 0·1 ml., were injected intradermally into depilated albino guinea-pigs,

with 2·0 μg. adrenaline (A) and without (S). The skin bearing the lesions was excised with a 2 mm. margin of normal tissue all round, tied to small pieces of perspex to prevent distortion during treatment, and fixed. After fixation the pieces were trimmed, dehydrated, cleared and embedded in paraffin, and serial sections (6μ) cut perpendicular to the skin surface through the entire lesion. All sections were mounted and every fourth slide stained with haematoxylin and eosin, each succeeding slide with a bacterial stain and intermediate slides according to the particular examination required. Both *Staph. aureus* and *Cl. welchii* were readily identifiable in sections stained with haematoxylin and eosin sections, or by Kirkpatrick's modification of Gram's stain. For the spores of *B. subtilis*, a modification of Armstrong's stain proved effective. Each variation in experimental conditions was tested in four lesions from two guinea-pigs.

With each organism, the S and A lesions 3 minutes old were almost indistinguishable. Spreading through the dermis from the site of the needle puncture (which in no case penetrated beyond the upper layer of the dermis) were irregularly shaped masses of carbon, which diminished in size towards the periphery of the lesion. In the centre they obscured the microbes by reason of their size and density ; at the periphery the masses were looser, and microbes could be distinguished. In the S lesions, microphages (polymorphonuclear leucocytes) were slightly more numerous in the capillaries lying between the panniculus carnosus and the subcutaneous fat, and predominated at the periphery of the vessels.

With *Staph. aureus* and *B. subtilis* at 30 minutes, there was general dilatation of capillaries and arterioles between the muscle and the subcutaneous fat in the S lesions, and the normally inconspicuous capillaries in the interstices of the fat and in the upper layers of the dermis were open. Microphages lined their endothelium and many had emigrated into the tissue spaces, and some were accumulating round the carbon and bacteria. At 1 hour these changes were more pronounced. Phagocytosis of the cocci and particularly of the spores was active, and of the carbon particles less so, and by 2 hours the *Staph. aureus* lesions were largely overrun by active microphages.

The A lesions were in sharp contrast. The 30 and 60 minute lesions differed little from the 3 minute ; a few microphages lay between the muscle and the fat at 2 hours, and large numbers at 3 hours ; but in no case did the migration equal that at 1 hour in the S lesion, nor did it extend, as in the S lesion, to the upper layer of the dermis.

With *Cl. welchii*, as with *Staph. aureus*, there was progressive migration of the microphages and active phagocytosis in the S lesions, and none in the A lesions until after 2–3 hours. The 1 and 2 hour S lesions showed also a thin zone of necrosis of connective tissue round the injection site, characterized by rounding and shrinkage of the cells and disappearance of thier cytoplasmic processes, and nuclear karyolysis. In the A lesion this necrosis was much more extensive, suggesting a much more intense intoxication ; fibrocytes were absent round the injection site ; and the nearby muscle fibres were invaded by bacteria, had lost striation and, in some cases, sarcous substance.

The alteration of tissue oxidation-reduction potentials.

The marked enhancement of infections by *Cl. welchii* and *Cl. septicum* (Tables III and IV) suggests that adrenaline, by cutting off the supply of oxygen, might

induce in the tissues a high reducing intensity favourable to the growth of anaërobic organisms. Accordingly two Eh indicator dyes, methylene blue and indigo disulphonate, were tested in the depilated skin of albino guinea-pigs, in the manner adopted by Fildes (1929) in his investigation of the *in vivo* germination of tetanus spores. Concentrations of 0·01 per cent methylene blue and 0·04 per cent indigo disulphonate proved to be most suitable for intradermal injection in 0·1 ml. volumes. Methylene blue in greater concentrations produced an intense and persistent staining of the superficial layers of the skin ; staining

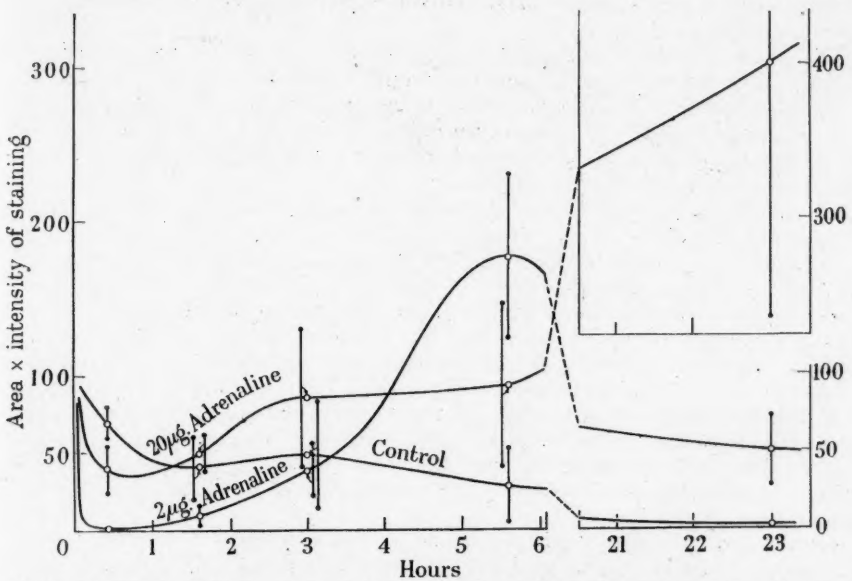

FIG. 3.—The course of staining of guinea-pig skin injected intradermally with methylene blue, with and without adrenaline. Each point represents the mean of readings from six guinea-pigs. The vertical lines indicate the standard deviation of the six observations.

by the 0·1 per cent solution faded from normal skin in 12–20 hours. The intensity of staining was estimated on an arbitrary, approximately logarithmic scale, which was checked by three independent observers against normal guinea-pig skin and filter papers, both stained by graded concentration of the dye. The total amount of staining in skin lesions was measured by intensity × area stained. In each test 2–4 animals were used, repeating the injection in different parts of their flanks and back. Fig. 3 summarizes the essential features of the results with methylene blue, which were similar in all tests made on a total of 35 guinea-pigs. Each point on the curves is the mean of six injection sites in two guinea-pigs. The scatter of values round the means is substantial, especially in the later readings, but there is no doubt that the three curves, for dye alone, and for dye

with 20 and 2 μg. adrenaline, represent three significantly different processes. Within the hour the colour of the control lesion declines, remains approximately stationary till the fourth hour, and, slowly fading, is invisible at 23 hours. With 2 μg. adrenaline there is almost complete or complete decolorization within 3 minutes, which persists for 30–60 minutes, after which the colour grows in intensity until at the fifth hour it is more intense than that of the initial lesion ; after this it fades, but is still marked after 23 hours. The same sequence occurs with 20 μg. adrenaline, except that decolorization is complete only at the periphery of the lesion, a small central spot of blue-green staining persisting throughout the whole period ; and the subsequent recolouring is intenser and more persistent.

By section of similar lesions at 30 minutes, staining of the whole thickness of the skin in the saline lesions and its complete decolorization by 2 μg. adrenaline was visible to the naked eye ; and with 20 μg. adrenaline the residual staining was confined to superficial layers of the skin. In fact, the staining in the 20 μg. adrenaline lesions at 5–23 hours was characteristic of a much stronger concentration of dye injected without adrenaline.

A similar sequence of events was observed in the skin of the rabbit's flank, and the mouse's ear.

It is clear, therefore, that the injected adrenaline lowers the Eh of the tissues to the level where methylene blue is decolorized. No such reversible decolorization could be demonstrated with 0·04 per cent indigo disulphonate (Fig. 4*a*). The dye disappeared within six hours, and the only effect of adrenaline was to prolong the period of staining. The stronger adrenaline lesions recoloured after what appeared to be a temporary decolorization. This was not a true decolorization, for the edges of skin cut through the centre of the lesion were well stained. The recolouring was apparently due to the removal of the injection fluid, which was to some extent masking the staining of the tissues ; for the reintroduction of 0·1 ml. saline into established 3-hour dye-lesions led to a similar temporary decolourization.

Because of possible errors in estimating the intensity of staining, the *in vivo* results were confirmed by tests in Thunberg's tubes. Skin from a newly killed guinea-pig was frozen in M/15 phosphate buffer, pH 7·0, cut in 20μ slices on a freezing microtome and made into a 15 per cent (wet wt./vol.) suspension with the buffer. At 37° C., both the suspension itself, and the supernatant fluid from centrifuged suspension, reduced 0·003 per cent methylene blue, but not 0·005 per cent indigo disulphonate. Adrenaline is known to affect tissue reducing systems ; low concentrations increase and higher concentrations diminish the reducing power of suspensions of frog muscle (Ahlgren, 1925 ; Euler, 1927). The same held for the suspension of sliced guinea-pig skin, as the following typical experiment shows :

Concentration of adrenaline μg./ml.	Reduction time (min). of methylene blue at 37°C.
333	83
33	54
3	36
0	42

It is possible, therefore, that adrenaline stimulates the reduction of methylene blue *in vivo*, apart from its vaso-constrictor action (Ball and Chen, 1933); and that the lesser *in vivo* effect of the higher concentrations tested (Fig. 3) were in part due to inhibitory effects of the kind exemplified above, in the test with 333 μg. adrenaline/ml.

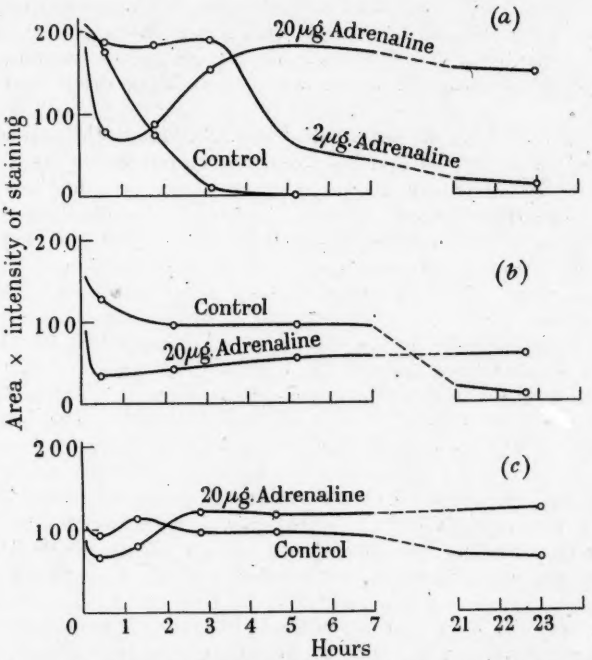

FIG. 4.—The course of staining of guinea-pig skin injected intradermally with various dyes, with and without adrenaline. Each point represents the mean of readings from at least three guinea-pigs. (*a*) Indigo disulphonate. (*b*) Trypan blue. (*c*) Pontamine blue.

The significance of increased reducing intensity in the tissues.

Whatever the mode of action of the adrenaline on the oxidation-reduction systems of the skin, the decolorization of methylene blue indicates a certain tissue Eh, whose magnitude will depend on the pH of the tissues. No exact measurements of pH were made, but tests in skin lesions 30 minutes old with brom-thymol-blue indicated pH levels of 7·0–7·2 in lesions made with 2 μg. adrenaline and with saline; and with phenol red, pH levels 7·0–7·2 in the adrenaline and 7·2–7·4 in the saline lesions. Even allowing for the errors in these colour estimations due to absorption of the dyes to tissue proteins, we may assume that the tissues pH lay well within the range 6·5–7·5. In this range, the 98 per cent reduction of methylene blue indicates an Eh of −0·023 to −0·058 volts, and of indigo disulphonate, an Eh of −0·148 to −0·197 (Fildes, 1929).

Since the skin tissue slices *in vitro* at pH 7·0 failed to attain the Eh (−0·1 volts) necessary for even the partial reduction of indigo disulphonate, it is probable that the Eh of the adrenaline-treated tissues *in vivo* was in the region of −0·04 to −0·1 volts.

It is problematic how far an Eh of this magnitude would aid the growth of aerobic organisms. Growing cultures of many pathogens attain Eh levels of this order and lower ; but as Hewitt (1936) points out, such reducing intensities may be the accompaniments of growth, and not necessarily conditions for its initiation and maintenance. Moreover, among the aerobes tested (Table III) there is no association between enhancement of infection by a bacterial species and its capacity to produce high reducing intensities in artificial culture. Thus, though *Bact. coli*, cultures of which can attain Eh values as low as −0·4 volts, is enhanced, so are *Proteus vulgaris* and *Ps. pyocyanea*, which do not attain low values in culture (Hewitt, 1936).

With regard to the clostridia, the Eh induced by adrenaline approaches the level of −0·2 volts, at which growth of these anaerobes starts, and may be expected to facilitate the attainment of this critical level as the infection develops. Nevertheless, we have little evidence that the diminution of Eh was a major factor in enhancing the clostridial infections.

Moreover, adrenaline has a marked effect on only two of the six clostridial infections examined (Tables III and IV) ; and we have observed some enhancement of *Cl. welchii* infections with 0·15 μg. adrenaline, an amount which induced no detectable decolorization of methylene blue, though, like larger amounts, it retarded the removal of the dye from the skin.

The fixation of dyes by adrenaline.

The fixation of methylene blue.—The retardation by adrenaline of the removal of methylene blue and to some extent of indigo disulphonate from the skin suggests that the enhancement of local infections might be due to a similarly retarded removal of injected bacteria. Immediately after injection, the removal of dye must in great part be due to fluid drainage ; after which removal by phagocytes presumably plays an increasingly important part. Adrenaline must affect the early stages of the removal, since even 0·15 μg., whose effect passes off with the hour, does so. It is pertinent to inquire what physiochemical effect adrenaline might have on the dispersion and retention of dyes and bacteria in the tissues.

At 37° C., strong adrenaline (1 mg./ml.) is a rapid precipitant of indian ink and slowly agglutinates washed suspensions, 10^9/ml., of *Cl. welchii*, *Staph. aureus* and *B. subtilis* spores ; but concentrations of 0·2 mg./ml. have little effect even after 12 hours either on ink or bacterial suspensions. Nevertheless, when a dilute solution of methylene blue was injected with adrenaline in this concentration into the skin of a transilluminated mouse's ear, the spread of the area of obvious staining round the site of injection observed under a low-power microscope was much less than that with methylene blue alone, as though the affinity of dye and tissues was enhanced. However, adrenaline in concentration 5–500 μg./ml. did not materially increase the readiness with which a suspension of fresh skin slices absorbed methylene blue from solution. Neither did adrenaline appear to act as a mordant ; for both dried sections of fresh skin, and filter

paper, were less readily stained by mixture of dye and adrenaline than by dye alone.

Adrenaline as a precipitant of tissue substances.—A possible mode of action is suggested by the observation that adrenaline precipitated extracts of finely sliced fresh skin. With a given extract, precipitation occurred only over a narrow range of adrenaline concentrations, and with dilution of the extract, the optimally precipitating concentration was shifted linearly to a corresponding lower concentration of adrenaline. Thus, in one test, 1 mg./ml. adrenaline optimally precipitated neat extract, 0·2 mg./ml. a 1/5 extract, and 0·04 mg./ml. a 1/25 extract. The precipitable material is evidently released when the skin is torn or sliced, for neither guinea-pig serum nor guinea-pig plasma was precipitated by adrenaline in concentrations ranging from 0·1 to 1000 μg./ml.

During injection into the skin of the adrenaline-bacterium mixtures, the tearing apart of the connective tissues by the injection fluid might liberate low concentrations of precipitable substance; and if adrenaline were absorbed preferentially by the tissues round the site of needle entry, the concentration of adrenaline in the advancing edge of the injection fluid might be low enough to precipitate this substance, with a consequent entrainment of bacteria in, or adsorption to, the precipitate.

The entrainment of bacteria in optimal adrenaline-skin extract precipitates was microscopically demonstrable *in vitro*. *In vivo* entrainment of this kind is obviously difficult to determine. But the following experiments demonstrate the possibility. A strip of filter paper 1 cm. wide is immersed for 0·5 cm. at its lower end in a reservoir of fluid under test, and the height climbed by the fluid is compared with that reached by substances in solution or in colloidal suspension. Changes in the absorption of the substance to the filter paper are inferred from the change in proportional height climbed. In this technique, which we modified from Bedson and Bland (1929), the filter paper represents the injected tissue, and the capillary climb the movement of injection fluid from the needle point outwards. Saline suspensions of washed living *Staph. aureus*, *Cl. welchii* and *B. subtilis* spores containing about 10^{10} viable cells per ml. were tested alone and mixed with adrenaline, and allowed to climb up strips of Whatman No. 1 filter paper. The paper was either untreated, or soaked in skin extract and dried rapidly *in vacuo* over P_2O_5. The extract was the liquid left after centrifuging a suspension in phosphate buffer of skin slices prepared as for the Thunberg tube experiments (p. 28). The height climbed by the bacteria was determined by cutting the strips into 0·5 cm. lengths, and pressing each firmly on to the surface of appropriate agar media. After 2 days' incubation *Staph. aureus* appeared as minute colonies in the filter paper, and the height climbed could be estimated to within 0·2 cm. The greater spreading power of *Cl. welchii* and *B. subtilis* colonies resulted in confluent growth over each section of strip, so that estimates were possible only to within 0·5 cm.

Up untreated paper, the climb of bacteria was not affected by adrenaline; but up treated paper, adrenaline markedly diminished it. Table VI summarizes a representative experiment. In repeated tests the percentage heights climbed varied with the different preparations of bacterial suspensions, and the particular batch of treated filter paper; and although the retardation of climb, indicating greater absorbability of the bacteria to the fibres of the filter paper coated with

extract, was usually greatest with highest concentrations of adrenaline (200 μg./ml.) tested, sometimes lower concentrations (40 μg./ml.) were more effective, again suggesting an optimally, not a maximally, effective concentration of adrenaline. These experiments demonstrate at least that adrenaline can alter the charge on tissue substances, making them readier to absorb, or be adsorbed to, bacteria.

The fixation of other dyes.—Though adrenaline-treated tissue substances may thus entrain bacteria in the experimental adrenaline lesions, it is unlikely that the holding of methylene blue in such lesions is due to such a process. As noted above, adrenaline did not mordant the methylene blue; if anything, it made it a less good tissue stain. Nor did it affect the climb of methylene blue up filter paper treated with skin extract; the percentage climb of the dye up the treated filter paper used in the experiment recorded in Table VI was

TABLE VI.—*Modification by Adrenaline of the Absorption of Bacteria to Filter Paper Treated with Extract of Guinea-pig Skin.*

Suspension.		Height (cm.) climbed by—		Percentage climb by bacterium.
Bacterium.	Concentration of adrenaline.	Liquid.	Bacteria.	
B. subtilis	*nil*	12·0	10	83
spores	20 μg./ml.	11·0	5·5	50
Staph. aureus	*nil*	10·0	4·5	45
	20 μg./ml.	10·0	2·0	20
Cl. welchii	*nil*	9·0	3·0	33
	20 μg./ml.	7·0	1·0	7

20 per cent both in the presence and absence of adrenaline. For comparison with the methylene blue lesions pontamine sky blue 6X and trypan blue, neither of them Eh indicators, were tested in guinea-pigs' skin. Curves, typical of tests on a total of 8 and 10 animals per dye, are included in Fig. 4 (*b* and *c*). The curves with 2 μg. adrenaline are not recorded; they were similar to the control, but at a lower level in the graphs. As with indigo disulphonate, 20 μg. adrenaline produces an initial drop in staining followed by an increase, which is presumably due, not to the reversal of a true decolorization, but, as suggested on p. 28, to an unmasking of colour as the injection fluid drains away from the injection site. Apart from this apparent decolorization, there is in each case a retardation of the disappearance of the dyes, most marked in indigo disulphonate, which disappears most quickly in control lesions, and least in trypan blue, which disappears most slowly in the control lesions.

This difference in behaviour is paralleled by the affinity of the dyes for skin tissue, which was determined with sufficient accuracy by mixing solutions of the dyes with suspensions of freshly sliced skin washed in saline, rapidly filtering in a Buchner funnel after a measured interval, and measuring the residual dye colorimetrically with due correction for the dye absorbed by the filter paper. Thus in 5 minutes a given suspension of slices removed 30 per cent of 0·02 per cent solution of methylene blue; and the corresponding percentages for equimolar solutions of the other dyes were indigo disulphonate, 33 per cent;

pontamine blue, 72 per cent; and trypan blue, 78 per cent. Moreover, when skin slices stained in this way for one minute by methylene blue and pontamine blue were immediately extracted with warm saline or guinea-pig serum at 37° C., only a trace of pontamine blue was recoverable, whereas a substantial amount of methylene blue could be extracted. But when the stained slices were held at 0° C. for 70 minutes, the methylene blue was fixed, for warm saline extracts contained only 20–40 per cent of the amount of dye extractable at one minute. In contrast, no visible dye could be extracted from pontamine blue-stained slices even after 5 minutes at 0° C.

In the light of these facts the persistence of methylene blue in the skin may be accounted for as follows: On injection, the dye is in the traumatic tissue spaces created by the injection fluid, and immediately begins to drain into the lymphatics. The tissues are stained, but at first this stain is partly removed—leached away—by the fluid constantly flowing from the blood capillaries to the lymphatics across the tissues. This natural flow of fluid is increased by the exudate that follows the endothelial damage by the saline solution of the methylene blue injected. The more irreversibly fixed portion of the dye is slowly removed by histiocytes and phagocytes, so that the lesion is colourless after 24 hours. On the other hand, when adrenaline cuts off the flow of fluid from capillaries to lymphatics, little leaching of dye takes place; a greater amount is firmly fixed to the cells, and persists longer in the lesion. With dyes of greater tissue affinity less leaching is in any event possible in the control lesions, and consequently less enhancement of staining under the influence of adrenaline.

The importance of these observations and the hypothesis derived from them lies in their application to bacteria. Quite aside from maintaining a supply of antibacterial substances and cells in the infected tissue, the inflammatory exudate may have a purely mechanical effect in carrying bacteria to the lymphatics before they become fixed to the tissues. If this were so, adrenaline, by inhibiting the flow, would ensure that more of the initially introduced bacteria were held in the local lesion.

The fixation of bacteria by adrenaline.

The hypothesis of local fixation of bacteria to the tissues by adrenaline was tested by comparing the numbers of bacteria, viable and total, in S and A lesions of various ages. Viable counts were made of extracts of whole lesions after cutting them into thin uniform sections. The lesions were made in the skin of guinea-pigs by washed suspensions of *Staph. aureus* 21 and *Cl. welchii* Type A. They were excised under chloroform anaesthesia, frozen in sterile M/15 phosphate buffer pH 7·0, containing 0·05 per cent liquoid (von Haebler and Miles, 1938), to inhibit any antibacterial substances from the blood. With sterile precautions, each lesion was cut into 20μ sections on a freezing microtome turned vertically, so that the sections fell directly into a sterile collecting tube. The sections from each lesion were suspended in a known volume of the liquoid buffer, subjected to a standard shaking, and a viable count was made of the resulting fluid extract. The A:S ratio indicates that at 2–3 hours the *Staph. aureus* counts are slightly and the *Cl. welchii* counts considerably higher in the A lesions (Table VII*a*). But the higher counts could be due, not to a fixation of the bacteria in the presence of adrenaline, but to a greater rate of killing in the S lesions by bactericidal

fluids and phagocytes. The contribution of such tissue bactericides to the result in the S lesion was estimated by measuring the bactericidal power of the animal's serum and blood, representing the source of many of the bactericidal elements in the inflammatory exudate. A sample of blood was taken from each of the guinea-pigs tested half an hour before the intradermal injections, and defibrinated. The samples were pooled, part of the pool was spun to obtain serum, and the

TABLE VIIA.—*The Number of Viable Bacteria Extracted from Infective Lesions in the Skin of Guinea-pigs formed with 2 μg. adrenaline (A), and without (S).*

Bacterium.	Guinea-pig number.	Age of lesion (min.).	Number of viable bacteria from lesion. A	Number of viable bacteria from lesion. S	Ratio. A:S
Staph. aureus	1	120	$16{\cdot}0 \times 10^3$	$4{\cdot}8 \times 10^3$	3·3:1
	2	150	$18{\cdot}8 \times 10^3$	$3{\cdot}1 \times 10^3$	6·3:1
	3	180	$13{\cdot}3 \times 10^3$	$4{\cdot}7 \times 10^3$	2·8:1
Cl. welchii	4	130	$10{\cdot}0 \times 10^5$	$2{\cdot}3 \times 10^3$	430·0:1
	5	160	$5{\cdot}0 \times 10^5$	$4{\cdot}0 \times 10^3$	125·0:1
	6	200	$10{\cdot}0 \times 10^5$	$1{\cdot}3 \times 10^3$	769·0:1
B. subtilis (spores)	7	150	81×10^3	56×10^3	1·4:1
			56×10^3	8×10^2	7·0:1
	8	210	19×10^3	7×10^3	27·0:1
			78×10^3	13×10^3	6·0:1
	9	240	66×10^3	34×10^3	1·9:1
			69×10^3	26×10^3	2·7:1
„ „	10	2	19×10^4	47×10^4	0·4:1
	11	2	29×10^4	14×10^4	2·2:1
	12	35	14×10^4	6×10^4	2·3:1
	13	40	20×10^4	14×10^4	1·4:1
„ „	14	2	$9{\cdot}0 \times 10^4$	$13{\cdot}6 \times 10^4$	0·6:1
	15	30	$12{\cdot}6 \times 10^4$	$9{\cdot}3 \times 10^4$	1·3:1
	16	30	$8{\cdot}0 \times 10^4$	$6{\cdot}5 \times 10^4$	1·2:1

bactericidal power of serum and blood, for the suspension of living organisms employed in the skin injections, tested by the method of Miles and Misra (1938).

Table VIIB summarizes the results. Guinea-pig serum did not kill *Staph. aureus* in 2 hours at 37° C., but defibrinated blood killed about 90 per cent of the smaller inoculum. *Cl. welchii* was highly susceptible both to serum and blood, the two smaller inocula being destroyed, and the largest diminished by 97 per cent. Neither organism is therefore a good indicator of fixation, since both are probably destroyed in the exudate, which at 2–3 hours is abundant in the S lesions. Indeed, the parallelism between susceptibility to the bactericidal power of guinea-pig blood, and the degree to which adrenaline enhances infections by these two organisms (Table III), suggests that the enhancement might be

TABLE VIIB.—*The Bactericidal Power of Pooled Fresh Serum and Defibrinated Blood of the Guinea-pigs against the Bacteria used for Inducing Skin Lesions.*

Organism.	Guinea-pig number.	Number bacteria/ml. seeded into test mixture.	Number bacteria/ml. surviving after 2 hr. at 37°C. in—	
			Serum.	Blood.
Staph. aureus	1–3	2,250	2,460	2,250
		225	360	**38**
Cl. welchii		21,500	**1,050**	**700**
	4–5	2,150	**100**	**0**
		215	**0**	**0**
B. subtilis (spores)	7–9	500	550	600
		50	50	45

Significantly diminished counts are indicated in heavy type.

predominantly due to transient protection of the bacteria from the inflammatory exudate.

We may note here that adrenaline does not appear to affect the bactericidal power of the exudate directly. The bactericidal power of pooled defibrinated guinea-pig blood against *Staph. aureus* and *Cl. welchii* was unaltered in the presence of adrenaline in final concentrations of 8 and 80 μg./ml.

The spores of *B. subtilis* were selected for their resistance to these bactericidal effects, at least for the period of the experiments. A washed suspension of spores was heated for 2 hours at 56° C. to kill the few vegetative forms detectable in stained smears. It proved (Table VIIB) to be unaffected by defibrinated blood. But, though from this viewpoint *B. subtilis* spores are a suitable indicator organism of fixation, the A:S ratios of *subtilis* lesion counts at 2–4 hours (Table VIIA, guinea-pigs 7–9) were too variable to provide satisfactory evidence. All indicated fixation by adrenaline, but about half the ratios were of the same order as those from 2-minute lesions (guinea-pigs 10, 11, 14), which ranged from 0·4:1 to 2·2:1. If this range represents the experimental error in determining a ratio that on *a priori* grounds should be 1:1, the values at 30–40 minutes (guinea-pigs 12, 13, 15, 16) are not significant, except that, like those for guinea-pigs 7–9, all four of them are consistently greater than 1:1, indicating higher counts in the A lesions. But even supposing the counts in the S lesions were significantly lower, they might have resulted, not from relative absence of fixation, but from a number of instances of non-lethal ingestion of two or more spores by one phagocyte (i.e. plural phagocytosis), each intraphagocytic aggregate giving rise only to one colony in the count plate.

In fact, phagocytic aggregation of bacteria was obvious in stained sections of 30 and 60 minute lesions by *Staph. aureus*, *Cl. welchii* and by *B. subtilis* spores. In all three cases the S lesions appeared to contain fewer bacteria than the corresponding A lesions. Nevertheless, in the *aureus* and *welchii* lesions the observed aggregations were insufficient to account for the relative paucity of bacteria in the S lesions; but the *subtilis* S and A lesions at one hour contained approximately the same number of spores, and differed only in the preponderance of intracellular spores in the S lesion. In lesions 2 and 3 hours old the bacteria

were also more numerous in the A series, and here it was clear, especially after 3 hours, that numbers exceeded those originally introduced, and that multiplication *in situ* had occurred.

The experiments recorded in Table VIIA were repeated on other occasions with substantially the same results. We conclude from these crude estimates of bacterial numbers that plural phagocytosis was not wholly responsible for the diminution of S counts in early *Staph. aureus* and *Cl. welchii* lesions; that the bacteria were being removed by other means; and that both phenomena were inhibited by adrenaline. The experiments with *B. subtilis* are inconclusive. We tried also to measure fixation of these spores by counting the number of viable spores that escape in a given time from A and S lesions in mice. The S and A injections were made in the right and left ears respectively, and spore counts made of extracts of frozen sections of the corresponding cervical lymph nodes. Here again, though there were indications that more spores drained into the lymph nodes from the S lesions, the experimental error was too great to warrant a definite conclusion. Nevertheless, the method appears to be worth refining, and we are attempting to do so.

DISCUSSION.

Renaud and Miget (1930) described in rabbit's skin a marked enhancement of *Cl. welchii* infections by 100 μg. adrenaline; a lesser enhancement of staphylococcal and streptococcal infections; and an increased survival of *B. subtilis* in the skin lesions. Brocard (1940) confirmed the result with *Cl. welchii* in guinea-pigs; his strain by itself was feebly virulent, and though small doses killed when injected with adrenaline, the bacteria recovered from the cadaver had not increased in virulence. Our extension of Cooper's quantitative study of the adrenaline effect reveals that among the species studied, striking degrees of enhancement were confined to *Cl. welchii* and *Cl. septicum*, though substantial enhancement occurred with a number of aerobic organisms. The enhancement is due to bacterial proliferation; the degree of enhancement of toxins is relatively small, though, as Mason (1936) first demonstrated with *Cl. chauvoei* toxin, it may be substantial with certain exotoxins.

It must be emphasized that we have tested a small number of species, mainly in the skin and muscle of the guinea-pig, an animal for which few of the organisms were pathogenic in the sense either that natural infections are common, or that artificial infections with small numbers produce a progressive disease. Moreover, many of the results may be characteristic of the strain used, and not of the species, though with *Staph. aureus*, the enhancement was of approximately the same order in the eight coagulase-positive strains tested.

The mode of action of adrenaline was studied mainly with *Cl. welchii* and *Staph. aureus*, representing markedly and moderately enhanced bacteria; and with *B. subtilis*, which was chosen as a feebly enhanced organism whose spores were insusceptible to tissue bactericides for the first few hours after injection. Upon these infections, adrenaline appears to act as might be expected, namely, through its being a powerful transient vasoconstrictor. The enhancement is determined in the 2–3 hours' vasoconstriction induced by the doses used, and there is no evidence of any after-effect. The adrenaline ischaemia results in a

deficient supply of oxygen, blood fluids and blood cells, so that the reducing intensity of the tissue increases, and both the diapedesis of leucocytes and the fluid exudation characteristic of the inflammatory response are inhibited. The absence of exudate almost certainly entails absence of antibacterial substances, and may also entail absence of a fluid flow which would otherwise dilute the bacterial toxins and assist in the transfer of the injected material to the lymphatic capillaries.

Except for the striking difference in numbers of bacteria visible in early lesions with and without adrenaline, our direct evidence for the last mechanism is not good, owing to experimental difficulties we hope to overcome. If it proves to exist, we must recognize that in the early stages of infection, *dispersion*, not localization, of the invading bacteria is an effective defence. This view gains some support from the observations of Duran-Reynals (1935), that in rabbit's skin the minimal infecting dose of many bacteria may be made innocuous if it is dispersed over a larger area of skin by injection with spreading factor ; and by observations like those of Sobernheim and Murata (1924), who found that in guinea-pigs the subcutaneous minimal lethal dose of *B. anthracis* was smaller than the intravenous ; and of Lange and Gutdeutsch (1928), who observed the same phenomenon in mice with pneumococci, streptococci and pasteurellae. In these cases it appears that the immediate dispersion of the infective material throughout the potentially bactericidal tissues has for the animal a greater survival value than its localization. The matter requires investigation with organisms of varying virulence and different natural modes of attack ; but on *a priori* grounds there is a case for dispersion as an early defence mechanism and for the speculation that adrenaline may act by opposing it.

In addition to the consequences of vasoconstriction, a direct physico-chemical action of adrenaline, either on the bacteria or the tissues, cannot be excluded. Adrenaline is a precipitant of certain unidentified tissue substances ; it directly enhances the reducing intensity in preparations of fresh guinea-pig skin, and in high concentration it agglutinates bacterial suspensions.

The artificial adrenaline effect is achieved only by physiologically enormous concentrations, and though it may bear on the natural functions of adrenaline in resistance to infection, there is nothing in our results to indicate any immediate connection. Systemic effects of the intensity that results in local enhancement would require very large doses of adrenaline, and enhancement is likely to be practicable only in relatively dense tissues where the necessary degree of local vasoconstriction can be maintained for several hours. The inefficacy of adrenaline in influenza virus infections of mouse lung, and pneumococcal infections of the mouse peritoneum, is in accord with this view.

We have referred throughout the paper to enhancement of *infection*, to avoid the current ambiguities (Proceedings, 1947) about the term *virulence*. As the word is sometimes loosely used, in the sense of an increased efficacy of the parasite in the host, virulence is enhanced by adrenaline ; but in the more exact sense of an attribute of the parasite *per se* virulence does not appear to be affected. In this more precise sense, virulence is usually measured by a particular end-result in the host-parasite relationship ; and, given a reasonably uniform group of test animals, it is possible to say that one bacterial strain is more virulent than another. However, the demonstration that the strain isolated

from an animal dead of an adrenaline-enhanced infection was more virulent than the original strain (Brocard, 1940, *supra*) would not necessarily mean that adrenaline had directly affected the bacteria. The increase in virulence might have been due to an adrenaline-induced increase in the susceptibility of the tissues, which permitted the more virulent variants of the strain to survive and initiate infection. Moreover, absence of any increase in virulence after such passage would not exclude a temporary, direct action of adrenaline on the injected bacteria. We have not therefore attempted to compare virulence in this way. Our analysis of the adrenaline effect, though incomplete, nevertheless strongly suggests that adrenaline directly affects tissue susceptibility, and not the virulence of the parasite. In particular, we would stress the demonstrable inhibition of exudation and diapedesis of phagocytes by adrenaline, and the parallelism between the degree of enhancement of *Cl. welchii* and *Staph. aureus* on the one hand, and their susceptibility to blood phagocytes and the bactericidal substances of the serum on the other (Tables III and VII*b*). For these infections at least, it seems to us unnecessary to postulate any substantial alteration of bacterial virulence by adrenaline. In this respect adrenaline resembles mucin, but differs from it in that it does not protect the bacterium against the tissue defences, but inhibits their mobilization.

We investigated the action of adrenaline in the first place as a means of enhancing experimental infections for testing chemotherapeutic substances. In animals and with bacteria for which it is effective, it has the advantage of doing little but temporarily inhibiting a natural tissue response ; and in this it is a marked contrast, for example, to $CaCl_2$, which acts only in concentrations that grossly damage the tissues. But its value for this purpose is limited to a few species ; and though tests of other pathogenic bacteria may reveal useful degrees of enhancement, the transience of the adrenaline effect means that enhancement will be confined to those infections for establishment of which the first few hours of the inflammatory response are critical.

SUMMARY.

1. The injection into the skin or thigh muscles of guinea-pigs of 2 μg. adrenaline renders these tissues more susceptible to some microbial infections. The resulting enhancement of infection by strains of various pathogens tested was as follows : *Cl. septicum* and *Cl. welchii*, 100,000-fold ; *Bact. coli*, *Pr. vulgaris*, *Ps. pyocyanea* and *Str. pyogenes*, 100- to 10,000-fold ; *C. diphtheriae* and *Staph. aureus*, 10- to 100-fold ; *B. subtilis*, *Br. abortus*, *Cl. oedematiens* and *Str. pneumoniae*, about 10-fold ; *Sh. flexneri*, *Sh. sonnei* and *V. cholerae*, 2- to 5-fold. Infections by strains of *Cl. histolyticum* and *Cl. tetani* were not enhanced. In the skin of the rabbit, *Staph. aureus* was enhanced 100-fold, vaccinia virus 10-fold and *H. pertussis* not at all.

2. Adrenaline chiefly enhances infection, not intoxication. Bacteria-free toxins were slightly enhanced. Two- to five-fold enhancement of crude toxins of *Cl. histolyticum*, *Cl. oedematiens*, *Cl. septicum* and *Cl. welchii* was observed.

3. The ischaemia induced by 2 μg. adrenaline leads to a diminution of tissue Eh and an inhibition of inflammatory diapedesis of leucocytes and exudation of blood fluids. These effects last for about 2 hours, and there is no detectable after-effect on the susceptibility of normal tissue to infection.

4. The temporary failure of mobilization of bactericides in adrenaline-treated tissue appears to be the chief factor in the enhancement of infections by adrenaline. There is some evidence of an early removal of bacteria from the injection site which is retarded by adrenaline, with a consequent increase in the severity of the local lesion. The removal of certain vital dyes is also retarded by adrenaline.

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THE PATHOLOGY OF PHENYLDICHLOROARSINE POISONING IN RABBITS.

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PHENYLDICHLOROARSINE is a vesicant resembling lewisite in its general action. Application of large enough doses to the skin may result in death. With a view to establishing the major effects contributing to the lethal action of the vesicant and the way in which systemic poisoning is brought about, a microscopic examination of the various organs was carried out.

METHODS.

The rabbits used were of both sexes. Before applying the vesicant, an area on the back was freed from fur by clipping as closely as possible with scissors,

The phenyldichloroarsine (obtained from the Ministry of Supply) was redistilled *in vacuo* to give an almost colourless product. In several experiments the vesicant was measured from a micrometer-controlled syringe to the ground end of a glass rod and then applied to the skin. This method of application was reasonably accurate (error ± 10 per cent.), but was tedious and tended to spread the vesicant on the skin. Latterly, the "microburette" developed at Porton (Lane, 1947) was used exclusively and found entirely satisfactory. Tissues for microscopic examination were fixed in Bouin's fluid, and paraffin slices about 7μ in thickness were cut. In the majority of cases contrast staining was carried out with azo-carmine, aniline blue and orange-G. The haemalum-eosin method was used in a few cases. Bone-marrow was stained by the method of May, Grünwald and Giemsa.

RESULTS.

The dosage and the interval before killing the animal are shown in Table I.

TABLE I.—*The Dosage of Phenyldichloroarsine and Period before Death of Rabbit.*

Rabbit No.	Method of application.	Dose mg./kg.	Fate.
7	Microsyringe	3·0	Killed after 1 hour
8	,,	6·0	,, ,,
10	,,	6·0	,, 4 hours
11	,,	6·0	,, 24 hours
13	,,	3·0	,, 41 hours
14	,,	3·0	Died after 70 hours
17	,,	2·4	Killed after 7 days
18	,,	3·0	,, 9 days
23	Microburette	5·0	,, 3 days
24	,,	5·0	,, 24 hours
25	,,	5·0	Died after 5 days
26	,,	5·0	,, 40 hours
27	,,	10·0	,, 48 hours
28	,,	10·0	,, 40 hours
29	,,	3 × 4·6 within 24 hours	Killed 24 hours after first application
30	,,	5·0	Killed after 24 hours
31	,,	4·0	,, 24 hours

Skin.—In the contaminated area marked injury to the epidermis, corium and subcutaneous tissues was observed (Rabbits 7, 23, 25, 26 and 27). The initial change appeared to be coagulation necrosis, involving the epidermis and cutis in the centre, surrounded by a large area of subcutaneous oedema. In the necrotic patch there was damage to the capillaries, lymph space and probably nerve fibres.

The epidermis became loosened without significant blister formation, and the corium showed dilatation of the capillaries and immigration of leucocytes.

Least damage was seen in the hair follicles, which appeared to be resistant to the vesicant to some extent.

The skin changes set in after 1 hour (Rabbit 7) and reached a maximum in 2–3 days (Rabbits 23, 26 and 27). By the fifth day (Rabbit 25) there were signs of repair in the peripheral area. As usual, repair of the damaged epithelium progressed by epithelial proliferation, particularly from the hair follicles, and the repair of the corium by granulation of the connective tissue with fibrosis. The scab covering the necrotic area showed no tendency to separate during the first week of observation.

Circulatory system.—In all the tissues examined the capillaries were seen to have become dilated and more permeable, while some organs (lungs, liver, kidneys and adrenals) contained interstitial and parenchymatous haemorrhages, and certain serous surfaces and cavities showed widespread petechiae.

There were no detectable pathological changes in the myocardium (Rabbits 7, 8, 10, 14, 18, 23 to 29) or the walls of large blood vessels. Veins, however, were always much dilated and full of blood.

Respiratory tract and lungs.—The only lung changes seen after 1 and 4 hours (Rabbits 8 and 10) were active hyperaemia and haemorrhage. Pulmonary oedema (Rabbits 11, 24 and 29) was observed 24 hours after the application of the vesicant. After 40 to 48 hours hyperaemia, haemorrhages and oedema appeared not only in the lung (Rabbits 13, 26, 27 and 28) but also in the peribronchial tissue, the changes being most marked in lower parts of the cartilage-free bronchioles. Bronchioles were filled with oedema-like fluid and blood. The epithelium of the bronchioles began to show signs of degeneration and sloughed into the lumen. Changes similar to those in the bronchioles were seen in cartilaginous bronchii.

The hyperaemic areas of the lungs were filled with haemorrhages, the alveoli were partially destroyed, and a process of mild, diffuse inflammation was seen. Deposition of fibrin was not appreciable. Compensatory emphysema was noted around the oedematous areas, most marked near the margins of the lobule. The capillaries of the alveoli were tortuous and engorged with blood. There was accumulation of fluid in the interstitial tissue of the alveoli which also contained some leucocytes. The lumina of the alveoli contained fluid, desquamated epithelium and sometimes erythrocytes. Blood was seen in the alveoli when haemorrhagic inundation had occurred in the terminal bronchioles.

Veins in the lungs were much dilated and full of blood, and oedema was noted in the perivascular connective tissue.

After 3 days (Rabbit 14) to 5 days (Rabbit 25), the first signs of an inflammatory reaction became evident, resembling that seen in bronchopneumonia. The bronchioles and alveoli contained desquamated epithelium, fibrin, blood and a few mononuclear leucocytes. Hyperaemia was well marked. In contrast, in rabbit 23 absorption of oedematous fluid and a general picture of healing were seen 3 days after application of the vesicant. In Rabbit 18 no changes in the lungs could be observed after 9 days.

Duodenum, intestine.—Investigation of the digestive tract revealed no significant changes in any of the animals examined (Rabbits 7, 10, 11, 13, 14, 17, 23 to 29), except in that, as in other tissues, veins were dilated and full of blood.

Liver, bile-ducts, gall bladder.—Peribiliary hepatic necrosis (necrosis hepatis peribiliaris diffusa) was observed in all animals (Fig. 1 and 2). The changes

were well marked after 24 hours and were at a maximum 40–48 hours after contamination of the skin. Fatty infiltration was looked for, but was never appreciable.

After 1 hour (Rabbits 7 and 8) the sinusoids were enlarged and engorged with blood, but there was no distortion of the parenchyma or degeneration of liver cells. After 4 hours (Rabbit 10), hyperaemia was at a maximum and there was moderate oedema. Whole blocks of liver cells were seen to be segregated by enlarged, blood-filled capillaries or by accumulation of intercellular fluid (oedema). Some cells were vacuolated. Sinusoids and the portal and hepatic veins were much enlarged and packed with blood, and oedema was observed in the perivascular tissue. Distinct necrotic changes, situated in the periphery of the lobules close to damaged bile-ducts, were first seen after 24 hours (Rabbits 11, 24 and 29). Hyperaemia and oedema in the large bile-ducts and in the gall bladder were observed after 4 and 24 hours, and there were haemorrhages in the submucosa of the gall bladder. The epithelium in some bile-ducts and in the gall bladder began to show signs of the degeneration and desquamation after 24 hours.

The most obvious changes seen after 40 and 48 hours (Rabbits 13, 26, 27 and 28) were widespread focal necrosis situated at or originating from the periphery of the liver lobule Usually, when liver cells had been damaged, the nuclei had disappeared (karyolysis), but in some cases fragmentary or shrunk nuclei were observed (karyorrhexis or pyknosis). The cytoplasmic granules were not visible. The actual positions of destroyed cells could still be seen in the more resistant connective tissue framework. Sinusoids within and surrounding a focus of necrosis were considerably dilated and filled with blood. This appeared to be in part secondary to the shrinking of necrotic liver cells. The capillary plexus, branches of the portal vein, and sometimes the hepatic vein were much enlarged and packed with blood corpuscles. Thrombosis was often seen in branches of the portal vein, but seldom in branches of the hepatic vein. The endothelial cells of sinusoids and capillaries were practically normal, but in necrotic areas the stellate cells lining the lumina of sinusoids were reduced in number. Haemorrhages were evident around large and small bile-ducts. Bile-ducts within and around tissue lesions displayed in all cases well-marked damage, varying from epithelial degeneration to rupture of the walls or complete necrosis and disintegration. These changes occurred first in the peripheral, intralobular bile-ducts. Sometimes (e.g. Rabbit 27) primary degeneration and desquamation of the epithelium of interlobular ducts took place in the areas between quite undamaged liver lobules.

Within 3–5 days the foci of necrosis could still be recognized in the form of connective tissue scaffoldings where cells had been destroyed, but first signs of

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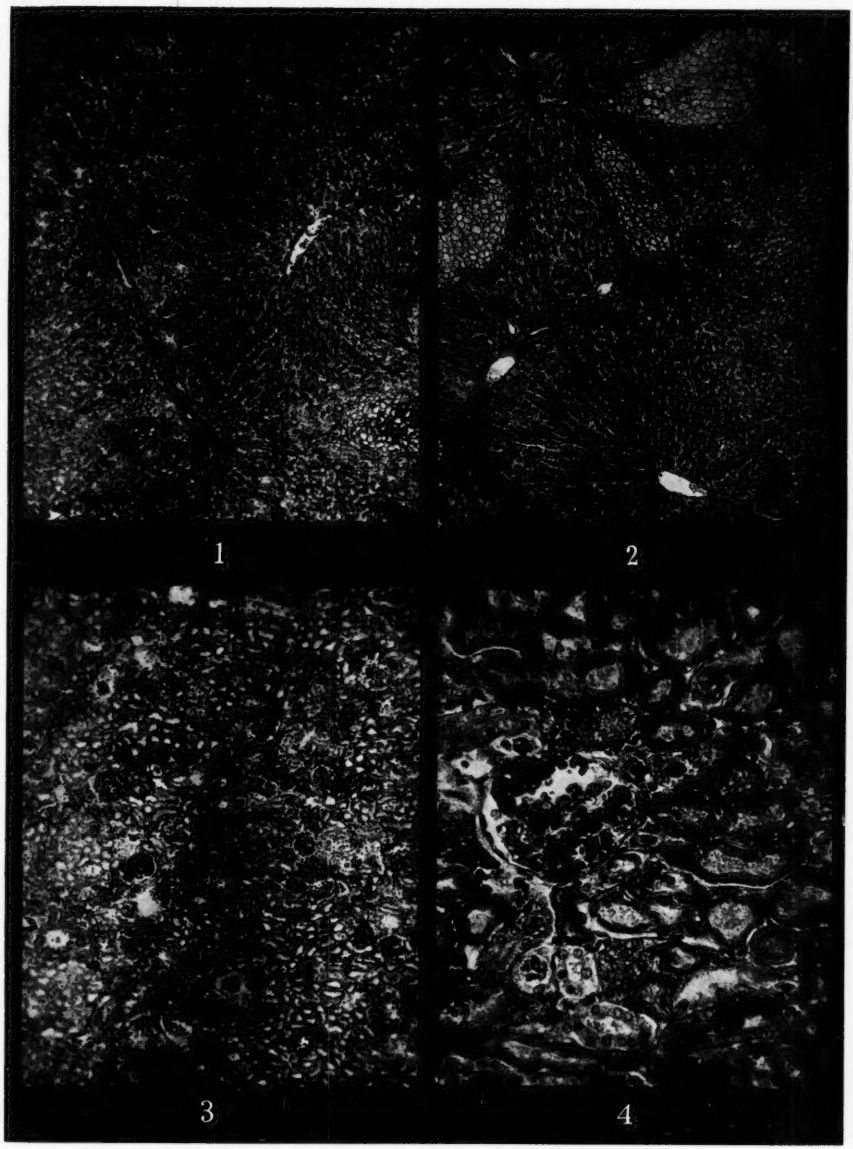
EXPLANATION OF PLATE.

FIG. 1.—Peribiliary hepatic necrosis. Rabbit 17, killed 7 days after 2·4 mg. of phenyldichloroarsine per kg. (× 48.)

FIG. 2.—Peribiliary hepatic necrosis. Rabbit 23, killed 3 days after 5 mg. of phenyldichloroarsine per kg. (× 48.)

FIG. 3.—Acute haemorrhagic nephritis and tubular damage in the cortex. Rabbit 14, died 3 days after 3 mg. of phenyldichloroarsine per kg. (× 48.)

FIG. 4.—Acute haemorrhagic nephritis and tubular damage in the cortex. Rabbit 14, died 3 days after 3 mg. of phenyldichloroarsine per kg. (× 240.)



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repair were evident (Rabbits 14, 23 and 25). Repair at this stage was mainly limited to proliferation of smaller bile-ducts and a fibroblast reaction, and occurred chiefly in Glisson's capsule which was markedly thickened and contained many new, small bile-ducts.

In Rabbit 17 which was killed 7 days after contamination of the skin the picture was similar, the reparative processes being particularly well-marked in the lobe to which was attached the gall bladder. The liver of Rabbit 18, killed 9 days after application of vesicant to the skin, showed no necrotic changes, although bile-duct proliferation associated with an interlobular fibroblast reaction was evident.

Kidney and ureters.—Histological changes in the kidney were more serious than was expected. Acute haemorrhagic glomerulo-nephritis was well-marked after 24 hours. After 40–48 hours, in addition to the glomerular changes, there was evidence of some tubular damage. In general, the picture appeared to be one of diffuse nephritis (Fig. 3 and 4). It seemed that damage to the renal tubules mainly involved the distal tubules in the cortex. There were no significant changes in the medulla of the kidney or in any of the ureters examined.

The kidney, apart from hyperaemia appeared normal 1 hour after contamination of the skin (Rabbits 7 and 8). At a period of 4 hours after poisoning (Rabbit 10), hyperaemia had increased and interstitial oedema could be observed. In another 20 hours, further to these changes, the first signs of glomerular damage became evident (Rabbits 11 and 24). After 40–48 hours from application (Rabbits 13, 26, 27 and 28) the most obvious change seen was the presence of fibrinous clots in the glomeruli with haemorrhages into the capsules and thence into the tubules. Disintegration of epithelial cells was well-marked. In the glomeruli, the haemorrhages and fibrin clots filled the capsule and thus obstructed the capillaries. Epithelial cells covering the capillary loops appeared to have been destroyed, and on occasion to have desquamated. The tubules (especially the second convoluted tubule), like the glomerular capsule, sometimes contained coagulated material (possibly albumen) and damaged epithelial cells. In the interstitial tissue there was marked accumulation of intercellular fluid (oedema), but no significant infiltration of white cells.

After 3 and 5 days (Rabbits 14, 23 and 25) hyperaemia was still considerable. The networks of capillaries surrounding convoluted tubules in the cortex were enlarged and packed with blood corpuscles. Also, but less frequently, the interwoven elongated capillaries in the medulla around the loops of Henle and the collecting tubules were affected. In Rabbit 23, under the capsule the venae stellatae connecting the renal and perirenal veins were enlarged and blood-filled. Three days after poisoning there became apparent a moderate degree of degenerative change in glomerular and tubular epithelium with haemorrhages and deposition of fibrin in the lumen of the capsule and the tubule.

In Rabbit 17, killed 7 days after the administration of the vesicant, there were small anaemic infarcts in the renal cortex (as well as infarctions of the adrenal cortex), resulting from the presence of an embolus in a branch of an artery, which in its turn penetrated the cortex. The infarctic mass had become dense, but the outlines of cells and even of nuclei could be made out quite clearly, except where glomeruli were completely necrosed and tubules had been totally destroyed and had collapsed. In the area of the lesion, where tubules had collapsed there

was compensatory enlargement of neighbouring tubules. Interstitial connective tissue showed a fibroblast reaction in this region.

Bone-marrow, spleen, lymph nodes.—Investigation of the red bone-marrow of two poisoned rabbits (30 and 31) revealed no significant changes other than small haemorrhages.

In two spleens examined (Rabbits 11 and 23) there appeared to be some damage to the malpighian corpuscles. Large mononuclear endothelial cells containing phagocytozed fragments of nuclear debris (lymphorrhexis) were seen in place of small leucocytes in nodules of lymphoid tissue. There were no significant changes in the splenic pulp or its cells. The endothelial mononuclear cells contained erythrocytes in various stages of disintegration.

Apart from marked oedema, no changes were seen in lymph nodes (Rabbits 26 and 27).

The histological picture made it clear that the cells of the reticulo-endothelial system (in the more exact sense, i.e. comprising endothelial cells of the spleen, certain branched cells in the bone-marrow, the stellate cells of Browicz-Kuppfer and the reticulum cells of the lymph nodes) were more resistant to the effects of phenyldichloroarsine poisoning than other cells.

Hypophysis, thyroid, parathyroids and adrenals.—The hypophysis (Rabbits 7, 24, 25, 27 and 28), thyroid and parathyroids (Rabbits 10, 23, 24 and 25) showed no changes apart from marked congestion.

The most characteristic alterations in the adrenals (Rabbits 7, 8, 10, 11, 17, 18, 23 to 28) were great congestion and haemorrhage. Blocks of partially damaged cells with pyknotic nuclei were separated by enlarged, blood-filled capillaries and accumulation of extracellular fluid. Occasionally, small thrombi could be seen. The intercolumnar spaces between the fascicular bundles were greatly dilated. Destruction of cells in the zona glomerulosa under the capsule was sometimes observed, and in one case (Rabbit 28), this had developed so far that foci of necrosis were present, in which even the outlines of cells had disappeared, leaving visible only outlines of nuclei in densely packed masses. Since the rabbit had died under the action of the vesicant these changes may have represented post-mortal autolysis. In some cases (e.g. Rabbit 17), infarctions of the adrenal cortex occurred in the area of the middle zona fasciculata. These did not extend to the outer zona glomerulosa and were clearly separated from neighbouring fascicular bundles. In the infarctic area, cells were partially necrosed, the network of vacuoles in the cytoplasm normally seen after fixation of sections having disappeared along with some nuclei. Outlines of the damaged cells and as a rule of the nuclei, could, however, be clearly seen.

DISCUSSION.

Many of the rabbits used in this work were examined for distribution and excretion of arsenic by Storey (1943) employing the method for arsenic determination of Levvy (1943). Interpretation of the results was complicated by the rather incomplete absorption from the skin found by Graham, Levvy and Chance (1947), but if allowance be made for this factor, the distribution figures resembled in many respects those for phenylarsenoxide (Chance, Crawford and Levvy, 1945),

the hydrolysis product of phenyldichloroarsine especially in the large and rapid uptake of arsenic by blood cells and lungs. Liver and kidney also showed high arsenic concentrations. In the case of phenyldichloroarsine, however, excretion of arsenic took place to approximately the same extent in urine and faeces, a total of about 2 per cent of the dose applied to the skin being cleared daily. The amounts of arsenic excreted during the first two days were no greater than at any other period, suggesting that absorbed arsenic was rapidly fixed in the tissues, from which it was released at a slow and steady rate. It may be noted that, although the concentration of arsenic in striated muscle was comparatively low, this tissue probably contained the greater part of the arsenic in the body, since it comprises nearly 50 per cent of the body weight of the rabbit.

The data for distribution and excretion of arsenic reflect the pathological changes observed. Since the necrosis at the site of contamination occupied only a small area of skin in these experiments, the local changes cannot be regarded as having contributed seriously to death. On the other hand, the central area of necrosis was surrounded by subcutaneous oedema over a very large area, the location of which seemed to be determined by gravity.

The more severe effects produced by phenyldichloroarsine appeared in general to result from the systemic action of the arsenic on the capillaries with consequent fluid loss from the circulation, and secondary damage which was mainly restricted to the lungs, liver and kidneys. In addition to secondary effects due to circulatory disturbance there appeared to be direct injury of certain organs, particularly the liver and kidneys.

After a period of 1–4 hours following the application of vesicant to the skin the capillaries throughout the body were seen to have become dilated. Numerous capillary haemorrhages, thrombosis, embolism and sometimes infarction were observed. The general picture of capillary paralysis with enormous accumulation of plasma in tissues, especially in the subcutaneous tissues and lungs, suggested that death at an early stage, i.e. 24–48 hours after contamination, was due to a shock-like condition, resembling that found by Cameron, Courtice and Short (1947) for lewisite poisoning.

The damage to the lungs (congestion and oedema) resulted mainly from disturbances in the circulation. Although foci of oedema were well-marked after 24 hours, there was no massive oedema such as is seen in phosgene poisoning. After 3–5 days the congestion and oedema disappeared or were followed by septic or aseptic complications.

The general picture of liver damage was one of necrosis in the periphery of lobules, close to damaged bile-ducts. The endothelium of the dilated and blood-filled sinusoids and the stellate cells seemed to escape injury. A clear-cut distinction cannot readily be made between the effects arising from disturbance of the circulation and those caused by a direct action of an arsenic derivative on the liver cells. It does not seem possible, however, that changes in the blood supply to liver cells (anaemic necrosis) could have been entirely responsible for the wholesale destruction which appeared at an early stage of poisoning. It seemed that peripheral necrotic damage of liver cells may have been associated with either changes in neighbouring sinusoids (distribution of arsenic derivatives *via* the blood stream) or in bile-ducts (excretion of arsenic derivatives). It has been shown that poisoned rabbits excrete as much arsenic in the faeces as in the

urine. Excretion in bile was not investigated by Storey, but the fact that there are no significant changes in the intestinal mucosa supports the view that arsenic in the faeces is excreted in the bile. It is not considered that the liver damage was sufficiently extensive at any time during the period studied (up to 9 days after contamination) to be itself a cause of the deaths seen in phenyldichloroarsine poisoning in rabbits.

In the case of kidney, it was again difficult to distinguish effects secondary to circulatory changes from direct damage to the renal cells. During the period of acute poisoning (4–24 hours) the effects of general and local capillary damage predominated. The oliguria, haematuria or even anuria regularly observed in the early stages of poisoning can be regarded as resulting from the shock condition (diminution in plasma volume, fall in blood pressure and, consequently reduction in glomerular pressure and glomerular filtration). In later stages (after 24–40 hours) it seemed that oliguria may have been due in part to direct damage of glomerular epithelium. The pathological changes were reminiscent of those seen in other forms of acute (haemorrhagic) glomerular nephritis. The blocking of the glomeruli with fused masses of erythrocytes and desquamated epithelial cells was sufficient to have caused diminution of urine filtration. Some tubular damage first appearing 24–40 hours after contamination was also probably in part due to direct poisoning, and may indicate that the distal tubules either actively excreted the arsenical in the blood or, alternatively, reabsorbed the toxic compound after it had passed through the glomeruli.

Changes observed in the adrenals (hyperaemia, haemorrhages, infarction) could all have arisen entirely from circulatory disturbances. It seems that the shock-like condition produced by phenyldichloroarsine was not secondary to damage of the adrenal cortex of the animals examined. In fact, one rabbit (17) was in good condition when killed 7 days after poisoning, although serious haemorrhagic infarction of the adrenal cortex had occurred.

The myocardium, digestive tract, bone-marrow, lymph nodes, hypophysis, thyroid and parathyroids showed no changes, apart from disturbances of the circulation. In the spleen, some lymphorrhexis was seen, suggesting that the toxic agent acted directly on this organ.

It is interesting to compare the changes caused by phenyldichloroarsine with the effects produced in animals by toxic doses of lewisite (Cameron, Courtice and Short, 1947) and therapeutic arsenicals, such as arsphenamine, neoarsphenamine, atoxyl and mapharsen (Petri, 1930 ; Gruhzit, 1934). All the compounds mentioned resemble phenyldichloroarsine in that they produce congestion, haemorrhages and other circulatory disturbances in all organs during the first 24 hours of poisoning.

The pathological changes seen with phenyldichloroarsine do not appear to differ from those observed in rabbits treated with lewisite, except in that the kidney damage may have been more severe in experiments with phenyldichloroarsine than has been described for lewisite. Phenyldichloroarsine, like atoxyl and mapharsen, produces much more serious kidney damage than is seen with arsphenamines (arsenobenzenes). Mapharsen is exceptional in that it affects the liver much less seriously than the other compounds under discussion. In general, all the therapeutic arsenicals act on other organs, such as the lungs, spleen and adrenals, in much the same way as phenyldichloroarsine. It thus

appears that the systemic changes produced by arsenical vesicants do not differ radically from those found with non-vesicant, organic, arsenic compounds.

After phenyldichloroarsine no perceptible injury to the tissue cells of bone-marrow has been observed. Bone-marrow changes have been seen in man and in experimental animals after repeated administration of arsphenamines. It should be borne in mind, however, that in the studies with phenyldichloroarsine the toxic agent was administered in a single dose.

SUMMARY.

1. After application of phenyldichloroarsine to the skin of rabbits, damage to the capillaries, and haemorrhages sufficiently severe to cause shock or early death, were seen all over the body, the effects being most pronounced in lung.

2. The histological pictures suggested that direct poisoning of liver and kidney occurred.

3. It was not considered that the liver and kidney damage was sufficiently extensive at any time during the period studied to be in itself a cause of the deaths seen in phenyldichloroarsine poisoning.

This investigation formed part of a research programme carried out for the Ministry of Supply under the direction of Dr. G. A. Levvy and a preliminary account of the work was given limited circulation by the Ministry in 1943.

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ANTHRANILIC ACID AS AN INTERMEDIATE IN THE BIOSYNTHESIS OF TRYPTOPHAN BY *BACT. TYPHOSUM.*

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ANTHRANILIC acid has recently been shown, by a number of workers, to be implicated in the biosynthesis of tryptophan by micro-organisms. Thus, Snell (1943) found that anthranilic acid, like indole, could replace tryptophan for the growth of *Lactobacillus arabinosus* and *L. casei* ; similar, but less detailed observations have been made by others (Greene and Black, 1943 ; Wright and Skeggs, 1945 ; Schweigert, Säuberlich, Baumann and Elvehjem, 1946 ; Schweigert, 1947). Snell (1943) postulated anthranilic acid as a precursor of indole in the synthetic chain leading to tryptophan, but was careful to point out that his results did not suffice to distinguish between this and the alternative, but unlikely, possibility that anthranilic acid was an intermediate in the synthesis of tryptophan from indole. More direct evidence in the case of the mould *Neurospora crassa* was afforded by Tatum, Bonner and Beadle (1944), who obtained, by X-irradiation, two mutants, one of which (10575) grew well on indole, but not on anthranilic acid, while the other grew well on both substances ; furthermore, they showed that anthranilic acid accumulated in relatively large amount in the medium in which the former of these two mutants was growing. These results clearly indicate that the (single) genetic block in the mutant 10575 was in the conversion of anthranilic acid into indole and thus establish that anthranilic acid is indeed the precursor of indole, at any rate in the case of this particular mould. Finally, Lemberg, Tandy and Goldsworthy (1946) identified anthranilic acid in small amount (*ca.* 2 mg./l) in the culture filtrate from *Bact. coli* grown both in the presence and absence of sulphathiazole ; in these experiments the possibility that the anthranilic acid was really a degradation product of tryptophan was not excluded.

The present work had as its objective the establishment of the role of anthranilic acid as an intermediate in the biosynthesis of tryptophan by *Bact. typhosum.* Two complementary lines of investigation were followed : First, the investigation of the accumulation of anthranilic acid in growing cultures of suitably modified, " trained," strains of the organism and, second, an attempt to find inhibitors which should be reversed specifically by anthranilic acid, indole and tryptophan.

THE PRODUCTION OF ANTHRANILIC ACID BY TRAINED STRAINS OF *Bact. typhosum.*

Three strains were used in this investigation, viz. 3390*a*, which will grow on a simple ammonia medium, 3390*d*, which will grow on an ammonia medium supplemented with indole or tryptophan, and 3390, from which the other two

were obtained by training (Fildes, 1945), which required an amino-acid medium with either indole or tryptophan. It was hoped that at least one of the trained strains would have a genetic block, between anthranilic acid and indole, leading to the accumulation of anthranilic acid in the culture medium.

Experimental Methods.

Since it was desired to follow the production of anthranilic acid in the medium during growth, it was necessary to devise some analytical method which could be applied to fairly small volumes of liquid while being sufficiently sensitive to enable concentrations of anthranilic acid of the order of 1 μg./c.c. to be estimated. It may be said at once that this objective has only been partially achieved and a really satisfactory method for the estimation of anthranilic acid is still required.

The first method investigated was that of Kotake, Otani, Nishino and Imai (1941), which depends on reaction with a modified Ehrlich reagent followed by oxidation with hydrogen peroxide ; in our hands the method was too insensitive and non-specific, and too much affected by small variations in the reaction conditions to be of any real value. The use of the first stage of the reaction, omitting the peroxide treatment (Tauber and Laufer, 1941) was more satisfactory but, although some preliminary unreported results were obtained with its aid, was too insensitive for convenience.

Recourse was finally made to diazotization and coupling with dimethyl-α-naphthylamine, the resulting red-violet colour being measured with an absorptiometer ; interference by non-acidic aromatic amines was avoided by first extracting such substances at pH 7·5 and applying the coupling reaction only to material extracted from the medium when subsequently brought to pH 3·8. *p*-Aminobenzoic acid gives a similar, and more intense, colour, but its presence is readily detected since the colour develops rapidly, in the course of a few minutes, whereas that from anthranilic acid requires several hours for development. As a final check, the dyestuff was extracted from the combined coupled solutions from each experiment and subjected to chromatography, following Lemberg, Tandy and Goldsworthy (1946). The detailed procedure was as follows :

An aliquot of the growing culture (usually 25 or 50 c.c.) was removed and autoclaved. Bacterial bodies were removed by centrifugation and the clear supernatant brought to pH 7·5 and thoroughly extracted with ether ; the residual aqueous layer was then brought to pH 3·8 with acetic acid and again thoroughly extracted with ether. This second etherial extract was dried over sodium sulphate and evaporated to dryness, finally for at least 24 hours in a vacuum desiccator over silica gel and sodium hydroxide. The residue was dissolved in N HCl (2 c.c.) and cooled in ice ; 10 per cent sodium nitrite (1 c.c.) was added and the mixture kept in ice for 30 minutes. Twenty-five per cent urea (2 c.c.) was then added and the mixture kept in ice for a further 30 minutes. Finally, 50 per cent acetic acid (4 c.c.) and 2 per cent alcoholic dimethyl-α-naphthylamine (1 c.c.) were added and the well mixed solution kept at 0–2° overnight. Next day, the colour developed was measured with the Hilger " Spekker " absorptiometer using 1 cm. cells and filters H503 ("Calorex ") and 605 (Ilford " Spectrum yellow-green "). The anthranilic acid concentration was then read off from a calibration curve, which was approximately linear up to a (final) concentration of 80 μg./c.c.; a reading of 0·200 corresponded to 20 μg./c.c. (final).

For chromatography the combined coloured solutions were treated with one-half to one-quarter their volume of M sodium acetate and the colouring matter extracted with ether. The ether extract was then shaken with 5 c.c. portions of 2 N sodium hydroxide, any colourless alkali extracts being discarded; the coloured alkaline extract was treated with M acetic acid (10 c.c.) and the dyestuff extracted with a little ether. This extract was then run, with gentle suction, through a column (200 mm. × 10 mm.) of activated alumina (B.D.H. "for chromatographic analysis") made up with 2 per cent acetic acid in ether, and developed with the same solvent. Contrary to the findings of Lemberg, Tandy and Goldsworthy (1946) we found that the anthranilic acid dyestuff gave a fast-running purple band (R = *ca.* 0·8) which was easily separable from the

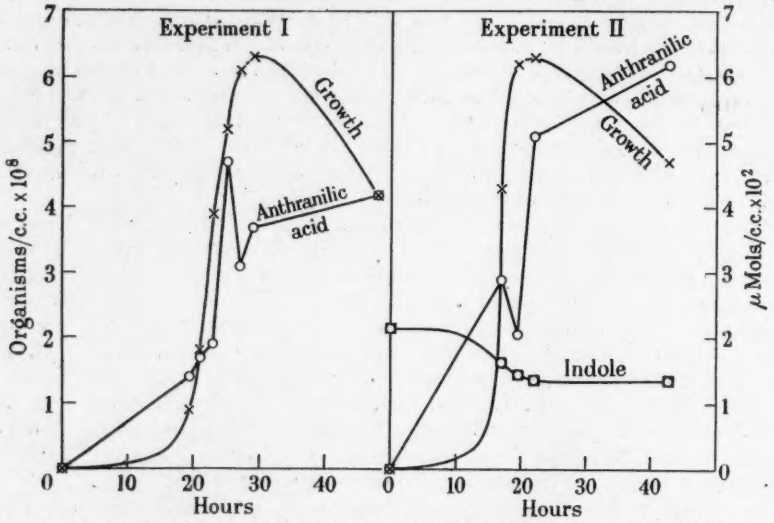


FIG. 1.—Production of anthranilic acid and utilization of indole; strain 3390*d*.

orange band of the *p*-aminobenzoic acid dyestuff which moved only about half as fast (R = *ca.* 0·4); the difference between these results, which were repeatedly confirmed, both by using the purified dyestuffs and by subjecting mixtures of the two amino-acids to the coupling procedure, and those of Lemberg, Tandy and Goldsworthy are no doubt to be ascribed to differences in the alumina used. Final confirmation of the identity of the products was obtained by mixing some of the eluate with an equal volume of a solution of the pure anthranilic acid dyestuff and chromatographing the mixture; failure to obtain two bands affords convincing evidence of the identity of the dyestuffs.

The organisms were grown, stagnant, at 37° in shallow layers in conical flasks (500 c.c. in a 2 l.; 300 c.c. in a 1 l.) in an atmosphere of air containing 5 per cent of carbon dioxide, from an inoculum sufficient to give, initially, 3,000 organisms/c.c. The media used were those of Fildes (1945), 3390 being grown

in the amino-acid medium in the presence of a final concentration of indole of M/50,000, 3390*a* in the ammonia medium, and 3390*d* in the same with the addition of indole to a final concentration of M/50,000. Bacterial growth was estimated by turbidity and indole with the Ehrlich reagent, after centrifuging, using in both cases the Hilger "Spekker" absorptiometer.

Experimental Results.

Strain 3390*d*. The results of two experiments are shown in Fig. 1; the fluctuations in the anthranilic acid values are no doubt to be ascribed to the inadequacy of the analytical method. In both experiments the dyestuff was extracted from the solutions after estimation of the colour and examined chromatographically; it proved to be homogeneous and inseparable from the authentic dyestuff prepared from pure anthranilic acid; the amount of *p*-aminobenzoic acid present in the solutions was certainly much less than 5 per cent of the total aminobenzoic acid present.

Strains 3390 *and* 3390*a*. The results obtained are shown in Table I.

TABLE I.—*Production of Anthranilic Acid by* Bact. typhosum 3390 *and* 3390*a*.

	Time (hrs.).	Organisms/c.c. $\times 10^8$.	Anthranilic acid µmols./c.c. $\times 10^2$.	Indole µmols./c.c. $\times 10^2$.
Strain 3390	0	0	0	2·1
	17·5	0·7	0·2	2·0
	19·5	1·7	0·2	1·5
	21·5	4·5	1·0	1·1
	23·5	6·2	0·7	1·0
	25·5	8·3	0·7	0·9
	27·5	8·9	1·3*	0·75
	29·5	8·4	1·0*	0·7
	48	6·4	1·2*	0·75
Strain 3390*a* (*no indole*)	0	0	0	
	22	0·8	1·0	
	24	1·0	0·8	
	26	2·5	0·8	
	28	5·3	0·7	
	30	5·7	0·7	
	32	6·1	0·6	
	36	5·2	..	
	54	5·1	0·8	
Strain 3390*a* (M/40,000 *indole*)	0	0	0	2·5
	20	0·9	1·6*	2·4
	23	3·0	1·5*	2·2
	26	5·7	1·1*	1·6
	28	6·4	0·4	1·4
	50	5·0	0·7	1·3

* Rapid colour development in these cases showed that much of the chromogenic material was *p*-aminobenzoic acid.

In a further experiment with strain 3390 two separate 500 c.c. batches were allowed to grow for 65 hours and then worked up as usual; colorimetric estimation in a final volume of 10 c.c. in each case, showed that the final concentrations of anthranilic acid in the two runs were 0·2 μmol./c.c. and 0·35 μmol./c.c. (0·3 μg./c.c. and 0·5 μg./c.c.); chromatographic examination showed the presence of much anthranilic acid accompanied by a small, but definite, quantity of *p*-aminobenzoic acid.

Discussion.

It is clear from these results that anthranilic acid is produced and excreted into the medium by all three strains of *Bact. typhosum* investigated. In only one case, however, viz. the partially trained strain 3390*d*, is the amount large. It is important to note that, in this case, the amount of anthranilic acid produced is very much greater than the amount of indole used up, the ratio of these two amounts, at full growth, being about 7·5:1. It is thus certain that the anthranilic acid cannot have arisen by degradation of indole and must therefore have been synthesized, but not used, by the organism. It seems, then, that the genetic block which distinguishes 3390*d* from 3390*a* and makes the former dependent on indole is between anthranilic acid and indole. The present results thus provide evidence in favour of the view that anthranilic acid is the precursor of indole in the biosynthetic chain leading to tryptophan.

The Reversible Inhibition of *Bact. typhosum* by the Methyl-Anthranilic Acids

The methyl-anthranilic acids were chosen for investigation in consequence of the earlier finding (Fildes and Rydon, 1947) that the methyl-indoles and methyl-tryptophans were potent inhibitors of *Bact. typhosum*, interfering with the utilization of indole and of tryptophan, respectively. As test organism it was necessary to use a strain which could use anthranilic acid and strain 3390*a* was adopted since the work outlined above showed that, of our three available strains, only this one could fulfil this requirement; it would have been better to have employed a strain which could use, but not synthesize, anthranilic acid, but it has so far not proved possible to obtain such a strain by training (private communication from Sir Paul Fildes).

Preparation of the Methyl-Anthranilic Acids.

N-Methyl-anthranilic acid was a specimen prepared by Dr. A. G. Lidstone with m.p. 178–179° (Wilstätter and Kahn, 1904, give m.p. 179°).

3-*Methyl-anthranilic acid* (3-methyl-2-aminobenzoic acid) was prepared by hydrogenating 3-methyl-2-nitrobenzoic acid (Müller, 1909) in alcoholic solution with Adams' catalyst. The product, after taking through acid to remove any unchanged nitro-acid, was recrystalized from water, forming needles, m.p. 168–169° (lit. m.p. 172°).

4-*Methyl-anthranilic acid* (4-methyl-2-aminobenzoic acid) was prepared by the method of Mayer and Günther (1930) and recrystallized from water, forming flat prisms, m.p. 174° (lit. m.p. 177°).

5-*Methyl-anthranilic acid* (5-methyl-2-aminobenzoic acid) was prepared from 5-methyl-2-nitrobenzoic acid (Müller, 1909) as described for the 3-methyl-compound and recrystallized from water, forming needles, m.p. 172–173° (lit. m.p. 172·5°).

6-*Methyl-anthranilic acid* (6-methyl-2-aminobenzoic acid) was prepared by the method of Gabriel and Thieme (1919) and recrystallized from benzene, forming leaflets m.p. 130–131° (Gabriel and Thieme, 1919, give m.p. 125–126°).

Methyl-Anthranilic Acids as Bacterial Inhibitors.

The test organism was *Bact. typhosum* 3390*a* and the tests were carried out as described by Fildes (1945) except that the inoculum (10,000 organisms per flask of 10 c.c.) was prepared by dilution of a 48-hour culture, the strength of which had been estimated by turbidity, using the " Spekker " absorptiometer.

In the first series of experiments (Table II) the five methyl-anthranilic acids were examined for their anti-bacterial activity, anthranilic acid itself being included as a control.

Attention may first be drawn to the inhibitory activity of anthranilic acid itself; this is most marked in the higher concentrations (M/100 and M/500), but some inhibitory activity persists down to concentrations as low as M/12,500. This finding cannot be regarded as inconsistent with the function of anthranilic acid as an intermediate in tryptophan synthesis since indole, which is generally accepted as an intermediate in this synthesis, was found by Fildes (1941) to be inhibitory at concentrations higher than M/4,000. The mechanism of this inhibitory activity of anthranilic acid remains to be elucidated; it is not markedly reversed by riboflavin (M/100,000).

Turning to the methyl-anthranilic acids it will be seen that two of these (the N-methyl- and 3-methyl-compounds) have inhibitory activities similar to that of anthranilic acid itself and one (the 6-methyl-compound) is completely non-inhibitory, while the other two (4- and 5-methyl-anthranilic acids) are much more inhibitory than anthranilic acid itself, the 5-methyl-compound being much the more potent inhibitor of the two. Further work was restricted to these two most inhibitory compounds.

Reversal of Growth Inhibition by 4- and 5-Methyl-anthranilic Acids.

The main object of the present work being to determine whether or not anthranilic acid was a precursor of tryptophan, experiments were carried out with four potential reversers of the inhibition, viz. anthranilic acid, indole, tryptophan and *p*-aminobenzoic acid, the argument being that, if anthranilic acid was in fact involved in the synthesis of tryptophan its reversing activity should resemble those of indole and tryptophan, rather than that of *p*-aminobenzoic acid which, so far as is known, plays no part in tryptophan synthesis. The results of these experiments are shown in Tables III and IV. The concentrations of the inhibitors were such as to minimize inhibition of the type shown by anthranilic acid itself.

TABLE II.—*Inhibitory Action of Anthranilic Acid and the Methyl-Anthranilic Acids.*

Inhibitor (Molarity × 10^6).	Growth in hours or days.*						
	22	28	48	3	4	5	7
Anthranilic acid							
10,000	0	0	0	0	0	++	++
2,000	0	0	++	++	++	++	++
400	0	Tr.	++	++	++	++	++
80	0	+	++	++	++	++	++
16	0	++	++	++	++	++	++
0	0	+±	++	++	++	++	++
N-Methyl-anthranilic acid							
10,000	0	0	+	++			
2,000	0	0	+±	++			
400	0	0	+±	++			
80	Tr.	+	++	++			
16	±	++	++	++			
0	±	++	++	++			
3-*Methyl-anthranilic acid*							
10,000	0	0	0	+±			
2,000	0	0	++	++			
400	0	0	++	++			
80	0	+	++	++			
16	0	+	++	++			
3·2	Tr.	++	++	++			
0	Tr.	+±	++	++			
4-*Methyl-anthranilic acid*							
10,000	0	0	0	0	0	0	0
2,000	0	0	0	0	0	0	0
400	0	0	0	0	++	++	++
80	0	0	++	++	++	++	++
16	±	+	++	++	++	++	++
3·2	+	+±	++	++	++	++	++
0	+	+±	++	++	++	++	++
5-*Methyl-anthranilic acid*							
10,000	0	0	0	0	0	0	0
2,000	0	0	0	0	0	0	0
400	0	0	0	0	0	0	0
80	0	0	0	0	0	0	0
16	0	0	0	++	++	++	++
3·2	0	Tr.	++	++	++	++	++
0	±	++	++	++	++	++	++
6-*Methyl-anthranilic acid*							
10,000	+±	++					
2,000	+±	++					
400	+±	++					
80	+±	++					
16	+±	++					
3·2	+±	++					
0	+±	++					

* In this and the other tables + signs are proportional to the amount of growth; Tr. = trace; ++ = full growth.

Inspection of Tables III and IV shows that anthranilic acid, indole and tryptophan are all effective in reversing the inhibition of the growth of *Bact. typhosum* 3390*a* brought about by 4- and 5-methyl-anthranilic acids; *p*-aminobenzoic acid, on the other hand, is ineffective in this respect. Since the inhibitory action of the methyl-anthranilic acids is reversed by both indole and tryptophan it is clear that these acids act by inhibiting some prior process in the synthetic chain leading through indole to tryptophan. That this inhibitory action is also reversed by anthranilic acid, but not by *p*-aminobenzoic acid indicates strongly that anthranilic acid is either the product or one of the reactants in the process inhibited by the methyl-anthranilic acids; it thus follows that anthranilic acid must itself be involved in the biosynthetic chain leading to tryptophan.

TABLE III.—*Reversal of Inhibitory Action of 4-Methyl-Anthranilic Acid.*

Reverser (2×10^{-5}M).	4-Methylanthranilic acid (Molarity $\times 10^6$).	Growth in hours or days.			
		22	28	48	3
Anthranilic acid	400	0	0	++	
	200	0	Tr.	++	
	100	±	+±	++	
	50	Tr.	+	++	
	25	Tr.	+	++	
	0	Tr.	+	++	
Indole	400	0	±	++	
	200	±	+±	++	
	100	±	+±	++	
	50	±	+±	++	
	25	±	++	++	
	0	Tr.	+±	++	
Tryptophan	400	0	0	++	
	200	0	Tr.	++	
	100	0	+	++	
	50	0	+±	++	
	25	+	+±	++	
	0	0	+	++	
p-Aminobenzoic acid	400	0	0	Tr.	++
	200	0	0	0	++
	100	0	0	++	++
	50	0	0	++	++
	25	0	0	++	++
	0	Tr.	+	++	++
None	400	0	0	0	++
	200	0	0	0	++
	100	0	0	+±	++
	50	0	0	++	++
	25	0	0	++	++
	0	0	+	++	++

TABLE IV.—*Reversal of Inhibitory Action of 5-Methyl-Anthranilic Acid.*

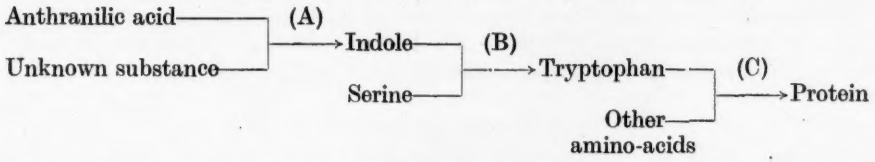
Reverser (2×10^{-5}M).	5-Methylanthranilic acid (Molarity $\times 10^6$).	Growth in hours or days.					
		22	28	48	3	5	7
Anthranilic acid	400	0	Tr.	++			
	200	0	++	++			
	100	Tr.	++	++			
	50	±	++	++			
	25	Tr.	+±	++			
	0	Tr.	+±	++			
Indole	400	0	±	++			
	200	±	++	++			
	100	±	++	++			
	50	±	++	++			
	25	±	+±	++			
	0	Tr.	±	++			
Tryptophan	400	0	0	++			
	200	Tr.	+±	++			
	100	Tr.	+±	++			
	50	Tr.	+±	++			
	25	±	++	++			
	0	0	±	++			
p-Aminobenzoic acid	400	0	0	0	0	0	0
	200	0	0	0	0	0	0
	100	0	0	0	0	0	0
	50	0	0	0	0	0	0
	25	0	0	0	++	++	++
	0	+±	++	++	++	++	++
None	400	0	0	0	0	0	0
	200	0	0	0	0	0	0
	100	0	0	0	0	0	0
	50	0	0	0	0	0	0
	25	0	0	0	++	++	++
	0	0	+±	++	++	++	++

The probable persistence down to low concentrations of some inhibitory action of the type shown by anthranilic acid itself makes it unwise to draw any further conclusions from these results. In particular it is not possible to decide whether the methyl-anthranilic acids act by interfering with the synthesis or with the utilization of anthranilic acid, and this question remains unanswered; the production of a strain of *Bact. typhosum* requiring anthranilic acid would go far towards solving this particular problem.

It would not be difficult to draw conclusions relating to the mode of combination of anthranilic acid with the bacterial enzyme involved in the reaction inhibited by the methyl-anthranilic acids, using arguments similar to those employed in the case of *p*-aminobenzoic acid (Rydon, 1947); the evidence, however, is slight and this aspect of the results will be fully discussed when experiments have been carried out with some other substituted anthranilic acids.

The Tryptophan Series of Biosyntheses.

The experiments described in this paper have afforded two independent lines of evidence leading to the conclusion that anthranilic acid is a precursor of indole in the biosynthesis of tryptophan by *Bact. typhosum* and, taken together, they may be regarded as establishing this point beyond reasonable doubt. We may summarize our present knowledge of the synthetic processes as follows :



In this scheme, process (A) is, probably, inhibited by the methyl-anthranilic acids, process (B) is inhibited by the methyl-indoles and indole-acrylic acid and process (C) by the methyl-tryptophans. The outstanding point now left for investigation is the nature of the substance which reacts with anthranilic acid to form indole.

SUMMARY AND CONCLUSIONS.

1. *Bact. typhosum* is capable of synthesizing anthranilic acid, not merely of producing it by degradation from indole or tryptophan.

2. 4- and 5-Methyl-anthranilic acids are potent inhibitors of the growth of *Bact. typhosum* ; this inhibition is reversed equally well by anthranilic acid, indole and tryptophan, but not by *p*-aminobenzoic acid.

3. It is concluded, from these two lines of evidence, that anthranilic acid is the precursor of indole in the biosynthesis of tryptophan by bacteria.

I am indebted to Sir Paul Fildes, F.R.S., the Director of this Unit, for much advice and encouragement, and to Miss B. W. Boughton, B.Sc., for the preparation of the methyl-anthranilic acids.

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IMMUNITY TO HOMOLOGOUS GRAFTED SKIN. III. THE FATE OF SKIN HOMOGRAFTS TRANSPLANTED TO THE BRAIN, TO SUBCUTANEOUS TISSUE, AND TO THE ANTERIOR CHAMBER OF THE EYE.

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SKIN autografts have long been known to survive heterotopic transplantation to almost any part of the body : brain, liver and spleen are as hospitable to skin grafts as skin itself. By grafting skin to regions of the body with major anatomical peculiarities—notably to the brain, which lacks a lymphatic drainage system, and to the anterior chamber of the eye, in which, as *in vitro*, grafts may be kept alive without penetration by blood vessels—the part played by otherwise inaccessible variables in transplantation immunity can be singled out and appraised. The use of heterotopic transplantation for such a purpose is far from new ; but earlier workers have sometimes failed to provide distinct answers to two quite distinct questions. Of skin homografts transplanted to the brain we may, for example, ask :

(*a*) Do such grafts survive transplantation to the brains of non-immunized animals ?

(*b*) Do they survive transplantation to the brains of animals which have been specifically immunized beforehand by graftings of tissue from the same donors to positions of known antigenic effectiveness elsewhere ?

It has been repeatedly shown—first by Shirai (1921) and Murphy (1926), and most recently by Tansley (1946)—that foreign homologous tissues grafted to the brain either do not provoke an immunity reaction or, if they do, do not respond to it. It will be shown here that they *do* respond to an immune state of proved effectiveness called forth by a preliminary grafting of foreign tissue elsewhere. The two questions, relating as they do to quite distinct immunological properties, may thus have contrary answers.

Earlier work gives a less decisive answer to question (*a*) when the site of transplantation is the anterior chamber of the eye. Greene (1940, 1942, 1943) has shown that even heterografts (notably of embryonic and tumour tissue) enjoy a relatively prolonged survival in the anterior chamber of the eye, and found that the anterior chamber of the other eye (or the testicle) became in due course resistant to further inoculations. Cheever and Morgan (1942), reporting on the use of the anterior chamber as a successful culture medium for the Brown-Pearce carcinoma, found that grafting to one eye did not prejudice the success of grafts later transplanted to the other. In experiments of the converse type, Saphir, Appel and Strauss (1941), confirming the observations of Besredka and Bardach (1936), showed that intra-testicular grafts of the Brown-Pearce carcinoma inhibited or altogether suppressed the growth of grafts later transplanted to the anterior

chamber. Apart from the inconsistency mentioned above, grafts to the anterior chamber seem thus to resemble grafts to the brain : they respond to, but do not elicit, an immune state. In this paper it will be shown with strict controls for each experiment that a skin homograft survives transplantation to the eye of a specifically and strongly immunized rabbit if, and only if, it remains unvascularized.

In addition, the experiments to be reported on below provide further evidence for the general rule (Medawar, 1945) that transplantation immunity is the outcome of a systemic and not a local reaction ; and they offer a rational explanation for the well-known clinical success of corneal homografts in human beings.

METHODS.

Full-grown rabbits weighing 2–2½ kg. from dealers' mixed stocks were used throughout. Save where some variant of the standard procedure is specifically mentioned, each individual test has made use of an independent pair of rabbits : a donor (*D*) and a recipient (*R*). (In one of the two groups of eye-implantation experiments, most of the recipients were linked together in pairs by sharing a donor between them. In these particular experiments the variable under investigation was the degree of vascularization of the grafts. As this is a function of operation technique and post-operative history, and not of the immunological relationship between donor and recipient, the procedure is unobjectionable.) Each recipient was immunized by the standard operation (Medawar, 1944, 1945) in which 8 large pinch grafts, each weighing modally 0·045–0·055 g., were cut from the thigh of *D* and accurately fitted to separate raw areas of the appropriate size on the skin overlying left side of the chest of *R*. Fifteen to 20 days after this heavy preliminary immunization, single further skin grafts from *D* were transplanted (*a*) to the centre of a 2 cm. × 2 cm. raw area stripped above the level of the panniculus carnosus from the skin overlying the right side of the chest ; and (*b*) to one of the following positions : the left cerebral hemisphere ; the sub-integumentary tissue between the panniculus carnosus and the body wall in the chest region ; and the anterior chamber of one eye.

In one group of experiments six days, and in the remainder ten days, was allowed to pass between this second operation and autopsy. The grafts were then examined *in situ* and fixed in formol-$HgCl_2$ for sectioning. The behaviour of the " orthotopic " control graft on the chest (i.e. the skin graft in skin position) provided a standard to which the behaviour of the others could be individually referred. In the majority of cases its long-standing necrosis stood witness to the completeness of immunization. But in some members of the six-day autopsy group (the time interval being chosen for this reason) the control graft showed some degree of partial survival. These experiments made possible a particularly accurate point-for-point comparison between the degree of survival of the control grafts and that of their contemporaries in anatomically unnatural positions elsewhere.

The control grafts, and those transplanted to the brain and below the integument, were rectangles ranging from 2 mm. × 3 mm. to 2½ mm. × 4 mm. in lengths of side, and so cut with a straight-edged scalpel blade as to include with the epidermis only the upper part of the compact connective tissue of the corium.

The best results from eye-implantation grafts were given by the use of squares of only 1 mm. in length of side, though larger grafts (2 mm. × 2 mm.) were used in earlier experiments not specifically reported on here. Very thin grafts of the Thiersch type are most easily cut from skin that has been rendered slightly turgid and hyperplastic by shaving three or four days beforehand, and this procedure was adopted alike for experimental grafts and controls in the great majority of tests. This preparatory treatment is objectionable only when mitotic counts in grafts of six days' standing are used to measure the degree of immunity (Medawar, 1946*a*).

To transplant a graft beneath the integument, a clean straight scalpel incision was made first through the superficial skin and then, avoiding any major skin vessel so exposed, through the panniculus carnosus. By inserting mosquito forceps through the incision horizontally (i.e. in the plane of the integument) and then slightly parting their tips, a tunnel was formed into which the graft could be tucked by a blunt probe, taking care only not to fold the graft upon its dermal surface. The initial incision was closed with a single suture clip.

The procedure adopted for transplantation to the brain was as follows : The full thickness of the skin was parted over a length of 5 mm. by a scalpel incision in the midline of the head following the parietal suture line. The skin—mobilized if necessary by cautious undermining—was then shifted in such a way as to carry the incision some 5 mm. towards the left side. The bony space underlying the incision in its new position was firmly scraped and then cautiously penetrated by a twist-drill 2 mm. in diameter. The membranes of the hemisphere being exposed, the graft was tucked into the bore of a standard tumour implantation trochar by folding it over on its cuticular surface and inserted vertically to a depth of 4–5 mm., whereupon the central plunger was thrust home. After withdrawal of the trochar the skin incision moved back to its original position medially and, after thickly dusting with sulphadiazine powder, was closed with two or three suture clips.

Transplantation to the anterior chamber proved to be a quick and easy operation : the cornea was penetrated at a point 2–3 mm. inwards of the limbus by a single stab with the tip of a very sharp ophthalmic scalpel. The graft was tucked through the slit so formed and manoeuvred into a central position by gentle strokes with a fine glass rod on the outside of the cornea. No special pains were taken to prevent the escape of the aqueous humour, which was fully regenerated in 24 hours.

Note on encystment.

Heterotopic skin grafts and cultures of skin in fluid media undergo or start to undergo encystment, the end-result being the formation of a closed vesicle.

Encystment may occur in one or other, or simultaneously in both, of two ways. The familiar dermal cyst of trapped skin epithelium is one in which the cuticular surface of the skin faces inwards and the basal layer faces outwards. Since the cuticle can be neither freed nor worn away, the characteristic appearance of such a cyst is an onion of cuticular layers bounded on the outside by a taut-looking layer of cells of the stratum germinativum. Such cysts, which are morphologically inside out, will be said to form "externally" (Fig. 7, 9, 13, 15). But in tissue cultures, and sometimes in brain and anterior chamber grafts, the

converse process happens: skin epithelium migrates over its own dermal substratum ("epiboly") and forms a cyst with cuticle outwards and basal layer inwards (Fig. 8, 11, 14). The histological examination of deep follicle epithelium and the thicker parts of the surface epithelium so entrapped shows that such grafts are often on the borderline of ischaemic degeneration when closure is complete. Cysts of this type will be said to form "internally." In this paper little turns upon the distinction between these two types of encystment, but it may prove to be of importance in interpreting the behaviour of homografts of cultivated endocrine gland tissue.

Grafts Transplanted beneath the Integument.

The experiments described in this section comprise six independent tests, each one making use of an independent pair of rabbits. Fifteen to 18 days after the grafting of the recipients with skin in high dosage from their respective donors, the immunizing grafts were found in all cases but one to have broken down. The recipients were then simultaneously regrafted with (*a*) a single control graft to a raw area on the skin of the chest, and (*b*) a graft of the same size, source and shape tucked beneath the panniculus carnosus. Examination *in situ* and autopsy were carried out six days later.

The control grafts showed almost complete survival in one recipient, various degrees of partial survival in three others, and total breakdown in the remaining two—a result unexpectedly faithful (in view of the small size of the sample) to the prediction based on the data of Medawar (1944), giving six days as the median survival time of "2nd-set" homografts.

All the grafts tucked beneath the integument were, or had earlier been, fully vascularized. Their apparent external encystment (Fig. 2, 4, 6) represents the outcome, not of active epithelial migration, but of the fact that the grafts were folded over on their cuticular faces before implantation. Apart from encystment, the six sub-integumentary grafts proved to be *identical* with their controls in the finest histological details; i.e. not only in the proportion and distribution of such epithelium as still survived, but also in the mode and degree of vascularization and vascular survival (the blood vessels in rabbit skin homografts being as a rule destroyed at the same time as the foreign epithelium) and in the pattern and intensity of the infiltration of the graft dermis by mesenchymal cells from the dermal vessels and graft bed. Three pairs of photographs illustrate this identity of response where the graft pairs show, respectively, full survival (Fig. 1, 2), partial survival (Fig. 3, 4), and total breakdown (Fig. 5, 6).

These experiments illustrate for the second time the general rule (Medawar, 1945) that the immune state called forth by grafting foreign homologous skin is systemic in compass, and not merely local; and, more particularly, they show that the survival of foreign epithelium is quantitatively uninfluenced by the presence or absence of native epithelium in the graft's neighbourhood. Cells of mesenchymal origin alone are present in sub-integumentary connective tissue.

Grafts Transplanted to the Anterior Chamber of the Eye.

The study of 48 single skin grafts transplanted to the anterior chamber makes possible the following general description of their crude morphology. Small

(1 mm. × 1 mm.) skin squares shift their position after implantation, and during the ten days allowed, as a standard procedure in all experiments, to pass before autopsy. At autopsy, the graft has been found either (*a*) weakly adherent to the corneal endothelium and free from other tissue and vascular attachments; or (*b*) attached more or less intimately to the pupillary border or the iris, or to its outer face in the angular space lying between the iris and the corneal endothelium; or (*c*) firmly imbedded in the anterior pole of the lens. Grafts ending in position (*a*) were never, and in position (*b*) were always vascularized. (On two occasions a graft attached to the corneal endothelium was connected to the iris by a delicate vascular pillar of "gathered up" iris tissue. These two grafts are, of course, classified under group (*b*).) Grafts found to be imbedded in the lens were unsatisfactory and showed ischaemic degeneration.

Whether vascularized or not, the epithelium of skin transplanted to the anterior chamber shows migratory and mitotic activity. A preliminary survey

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EXPLANATION OF PLATES.

FIG. 1–6.—Skin homografts six days after transplantation to a raw area on the chest (Fig. 1, 3, 5) and to a subintegumentary pocket (Fig. 2, 4, 6) on rabbits rendered immune by earlier graftings of skin from their respective donors. In the pair illustrated by Fig. 1, 2, the epidermis is almost wholly surviving and dermo-epidermal cleavage is no more than incipient. Fig. 3, 4, illustrate a low degree of partial survival, and Fig. 5 and 6, total breakdown. Encystment apart, the modes of response of the grafts in their two different positions are identical. All × 22.

FIG. 7.—A skin autograft which has become strongly adherent to and vascularized through the iris ten days after transplantation to the anterior chamber of the eye. Migratory activity of the epidermis has brought about complete external encystment, and sebaceous glands have formed anew. × 22.

FIG. 8.—As Fig. 7, but from a vascularized autograft seen in section at a level at which internal encystment is complete; the epidermis has abandoned its old cuticle and grown round upon its own dermis instead. × 22.

FIG. 9.—Contrast Fig. 7: a skin homograft ten days after transplantation to the eye of a specifically immunized recipient. The graft, intimately attached to the iris, has become vascularized and shows no trace of surviving epithelium. Note, however, that a high degree of external encystment had occurred before breakdown. × 22.

FIG. 10, 11.—A *non*-vascularized skin homograft ten days after transplantation to the anterior chamber of the eye of a specifically immunized rabbit (Fig. 11). The complete necrosis of the control graft on the chest (Fig. 10) is witness to the completeness of immunization. Although proliferation is subdued, migratory activity of the still fully-surviving epithelium has gone so far as to bring about an almost complete internal encystment. × 22.

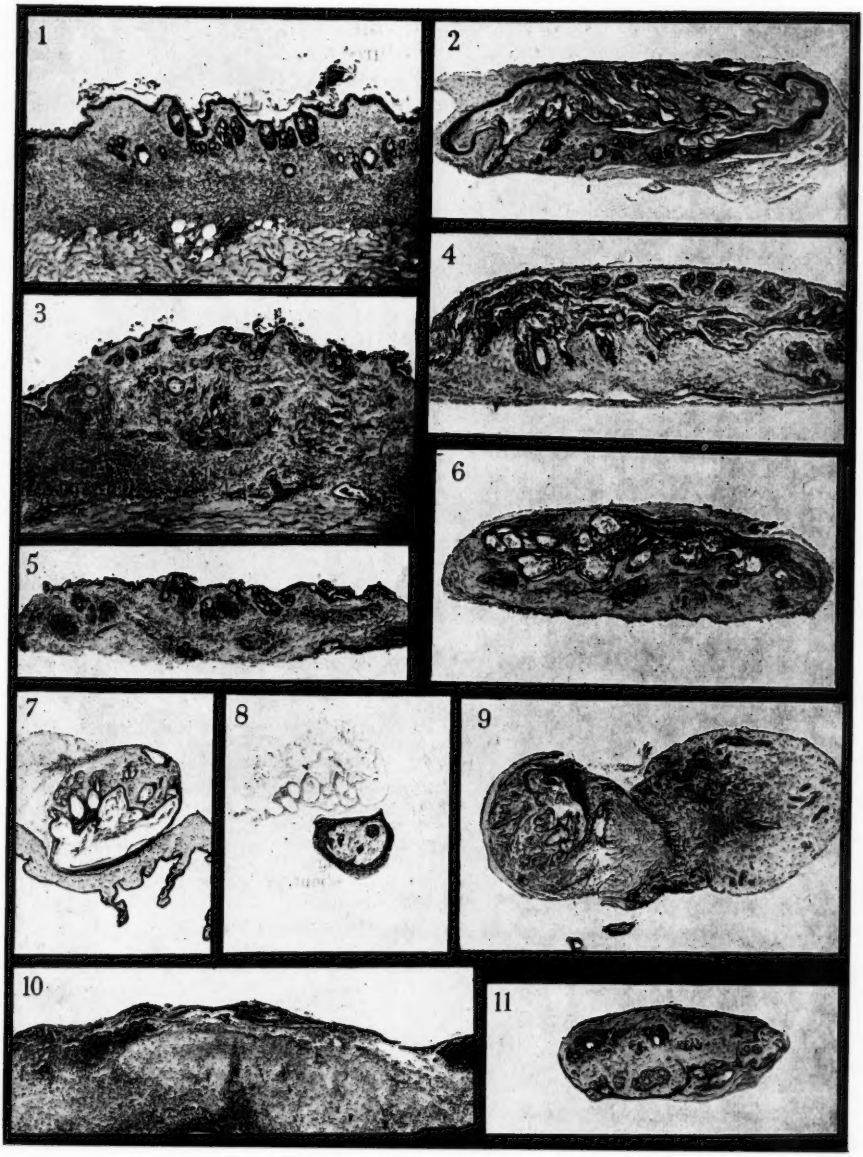
FIG. 12, 13.—As Fig. 10, 11, but from another pair of rabbits in which the surviving, non-vascularized homograft in the anterior chamber shows a mixture of internal and external encystment. × 22.

FIG. 14, 15.—Skin autografts ten days after transplantation to the brain. Note the vigorous epidermal proliferation, the rich population of dermal blood vessels, and the migratory activity of the epithelium, which has led to the beginnings of internal encystment (Fig. 14) or to a mixture of internal and external encystment (Fig. 15). × 22.

FIG. 16, 17.—Contrast Fig. 14, 15. Skin homografts ten days after transplantation to the brains of specifically immunized rabbits. Total breakdown of long standing. × 22.

FIG. 18, 19.—Illustrating the bare survival of a trace of epithelium at the margin of a skin homograft ten days after transplantation to the brain of a specifically immunized rabbit (Fig. 18); and the formation of isolated "cultures" of surviving skin epithelium in an almost wholly avascular region of the ground substance of the brain (Fig. 19); from a similar homograft which elsewhere has broken down completely. × 59.

All the photographs are of sections stained with Ehrlich's haematoxylin, aqueous orange-G, and alcoholic eosin.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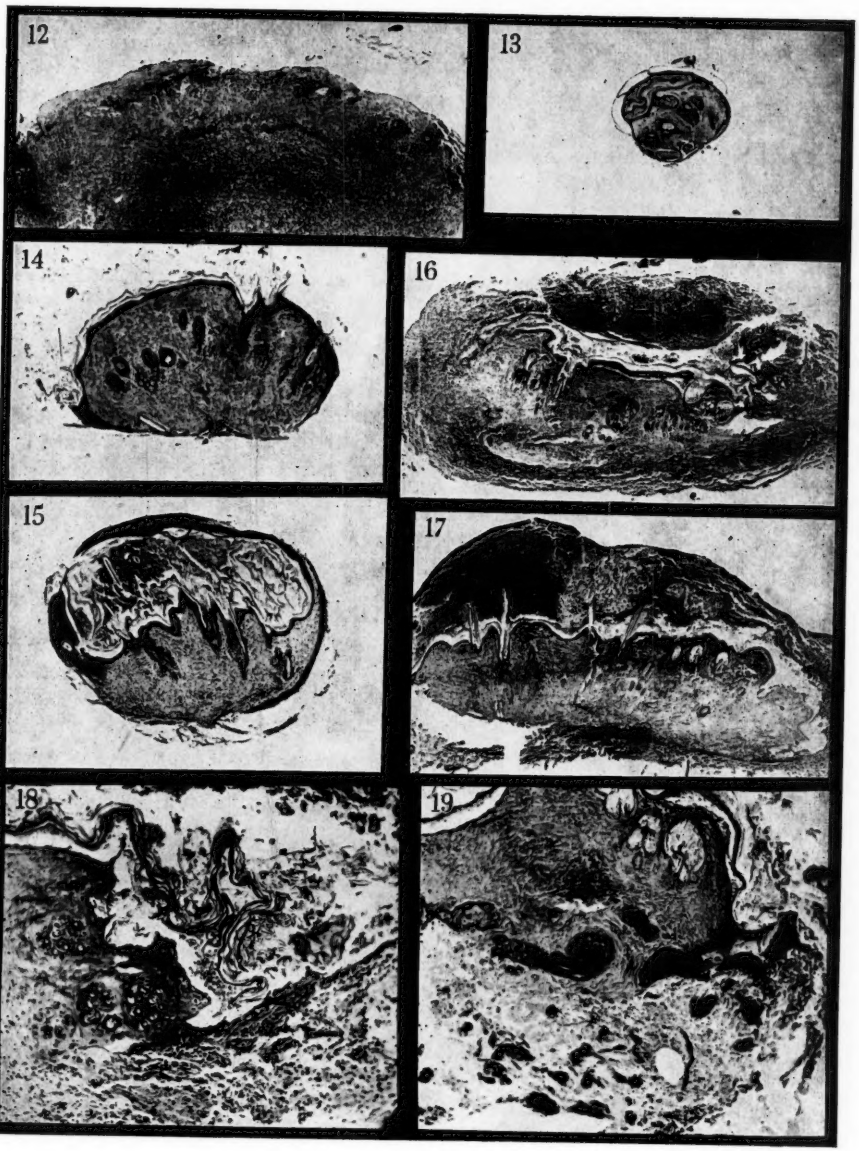
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of the growth of 10 independent autograft controls (Fig. 7, 8) showed that only three were vascularized after 10 days in residence. The two grafts with the greatest proliferation and migratory spread of epithelium—being also the only two to show new or persistent differentiation of sebaceous gland epithelium (Fig. 7) and the formation of hair follicle primordia—belonged to this group of three. Seven of the grafts showed full, and the remainder partial external encystment, accompanied in some cases by at least the beginnings of the migration of epidermis round dermis (Fig. 8). All the experimental grafts showed a similar preponderance of external encystment, and on this score there was nothing to choose between them.

Other autograph controls are mentioned in the report on the second experiment below.

First experiment.

This experiment comprises ten tests of the same form, each one making use of distinct but not independent donor-recipient pairs. (Two recipients had unique donors ; the remainder shared four donors between them by pairs.) The initial immunizing grafts were found to have broken down completely when inspected 15–17 days after their transplantation, and the recipients were thereupon simultaneously regrafted with (*a*) a single control graft to a raw area on the skin of the chest, as before ; and (*b*) a 1 mm. × 1 mm. square in the anterior chamber of the right eye. Examination *in situ* and autopsy were carried out ten days later.

(It should be mentioned here that, by contrast with sub-integumentary grafts, there is one unavoidable source of disparity between such eye-implantation grafts as are vascularized and their invariably vascularized controls on the skin of the chest. The control grafts are penetrated by blood vessels within 48 hours. of planting, but eye implantation grafts may wander about free for several days before forming adhesions to the iris border or elsewhere. This delay of vascularization is sufficient to explain the fact that vascularized eye grafts may lag slightly but perceptibly behind their controls when undergoing breakdown.)

In all cases but one the control graft on the chest showed total breakdown of long standing after 10 days, in accordance with expectation (Fig. 10, 12). In the one exceptional case the recipient's original donor had died, and, rather than abandon the experiment entirely, the recipient was regrafted from a new donor source. The survival of the control graft 10 days after transplantation to its thus non-specifically immunized recipient provides yet another demonstration of the strong donor-specificity of the immune reaction (Medawar, 1946*a*). The anterior chamber graft in this animal showed full survival in spite of rich vascularization—an exception which proves the rule to be formulated below.

The remaining nine grafts fell into the following groups : (*a*) three fully vascularized grafts, of which two showed total breakdown (Fig. 9) and the third a barely perceptible fraction of epidermal epithelium which could still be judged surviving on histological evidence (Medawar, 1944, 1947) ; (*b*) four grafts wholly devoid of vascular attachments which were in a fully surviving state. As with non-vascularized members of the set of 10 autograft controls, proliferation and migratory activity were subdued (Fig. 11, 13). Finally (*c*), two grafts which were found to be intimately and firmly adherent to the outer face of the iris, but

which failed to reveal the characteristic dilated and engorged capillary vessels on sectioning. Both showed partial survival of epithelium. It seems likely that these two represent an intermediate condition : late attachment to the iris and a correspondingly delayed and rudimentary penetration by iris vessels, with the consequence that breakdown, though in progress, was not complete at the time of inspection. A moderate leucocytic infiltration of these grafts showed that *some* effective vascular contact must have been established, despite the lack of overt evidence of the presence of blood vessels.

These experimental results show (as those to follow confirm) that *non*-vascularized eye implantation homografts are exempt from breakdown when transplanted to fully and specifically immunized rabbits. If allowance is made for the unavoidable disparities between their respective times of vascularization, they further show that eye implantation homografts penetrated by blood vessels, notwithstanding their minute size, participate fully in the specific degenerative changes undergone by their controls.

Second experiment.

It was thought worthwhile to repeat the preceding experiment as a whole with one modification : the single donor skin homograft tucked into the anterior chamber was accompanied by a graft of mesenchymal tissue from the recipient itself. Pairs of grafts so implanted form a composite ball of tissue which may or may not come to be vascularized.

The host's contribution to this compound graft was cut from the " active " (hyperaemic, turgid, rapidly proliferating) tissue of the bed of any one of the original immunizing homografts after their breakdown. The homograft was stripped from its bed and a cube of tissue 1–1½ mm. in length of side was snipped out from the middle of the vascular crater so exposed. One such cube was inserted with each donor skin homograft and gently manoeuvred to its side. The tissue of which it is composed is wholly mesenchymal, and consists of fibroblasts, histiocytes, miscellaneous leucocytes and vascular endothelium, supported by a delicate web of collagen fibres of new formation. (For a full description of the anatomy of the homograft bed, see Medawar (1944).)

In other respects, and with the added refinement that each recipient was independently paired with a single donor, the ten pairs of animals that comprise this experimental group were treated just like their predecessors. The recipients were regrafted from their respective donors 16–20 days after transplantation of the immunizing dose of grafts ; and at autopsy ten days later the control grafts on the chest showed total breakdown of long standing in all cases. Immunization was thus demonstrably complete.

The standard double implantation test, repeated ten times, consisted of transplanting *D* skin + *R* graft bed tissue to one eye of *R* after its specific immunization by tissue from *D*. To control against the possibility of some non-specific toxic action by (e.g.) an exudate from the inflamed graft bed tissue, and to assess the magnitude of any specific contribution of its own to its neighbour's fate, six of the ten recipients were caused to receive *R* skin + *R* graft-bed tissue in the other eye. The donors corresponding to three of these six recipients received, in addition, *R* skin + *R* graft-bed tissue in one eye, and *D* skin + *R* graft-bed

tissue in the other. In the outcome, eleven of the twelve control grafts, five of them unvascularized, were wholly autograft-like and showed vigorous proliferative and migratory activity. The twelfth had become imbedded in the anterior pole of the lens and, like other such grafts, showed non-specific ischaemic changes. It may be concluded that any action attributable to the graft-bed component of the compound eye implantation graft is specific in character.

All ten experimental compound grafts gave evidence of migratory activity by the skin epithelium ; predominantly, as before, of external encystment, but in some cases trivially complicated by the tendency of *D* epithelium to abandon its own cuticular investment and to grow round the *R* graft-bed tissue component. Fibroblastic cells in the *R* component survived and proliferated and showed some tendency to form a fibrous capsule round the dermal remains of such *D*-grafts as, having been vascularized, had broken down.

As with the first experiment, the ten compound grafts were found at autopsy to fall into three groups : (*a*) a group of six, totally broken down, of which five had heavy iris adhesions and were overtly vascularized, while the sixth had a fine iris attachment with only leucocytic infiltration to give evidence that vascular penetration had in fact occurred (see above). Two grafts, (*b*), were adherent to the corneal endothelium and unvascularized : these showed full epidermal survival ; and (*c*) the remaining two showed partial survival and a weak marginal vascularization. As with group (*c*) of the preceding set of ten, these two are taken to represent grafts which had anchored themselves late to the iris and which lagged proportionately behind their controls in submitting to the immune reaction.

This second experiment therefore reinforces without enlarging upon the results of the first : grafts that are not vascularized are not appreciably affected by the recipient's specific immune state. When any appropriate allowance is made for inequalities of vascularization time, grafts that *are* vascularized submit to the immune reaction in the fashion of their orthotopic controls. The addition of mixed mesenchymal tissue from the bed of one of the recipient's broken-down homografts was without appreciable effect, at least in the dosage relationship of host to donor tissue here used.

Grafts Transplanted to the Brain.

In these experiments only one problem was at issue : whether homografts transplanted to the brains of specifically immunized rabbits survive, or submit to the immunity reaction and break down.

A series of six 10-day autograft controls showed brain tissue to be an admirable culture medium for grafted skin : the dermis of such grafts is richly vascularized, and this enables it to support a deeply stratified and strongly proliferating epithelium which encysts internally (Fig. 14) or externally or in both ways simultaneously (Fig. 15).

In the experimental series a graft was transplanted to the left cerebral hemisphere and simultaneously, as usual, to a raw field on the chest, 16–20 days after the preliminary specific immunization. Immunization was effective in all cases, and the orthoptic control graft on the chest became completely necrotic in the ten days allowed to pass between transplantation and removal at autopsy.

Four of six brain grafts so implanted likewise showed the characteristic

appearance of total breakdown of long standing (Fig. 16, 17). The cells of the graft epidermis, with pyknotic nuclei and strongly eosinophilic cytoplasm, had broken away from the dermis and in places were undergoing maceration. The dermis, destitute of any living element, was either clear or lightly peppered with twisted chromatic fragments representing the nuclear remains of leucocytes which had invaded the graft before, and during the earlier stages of, breakdown. Lymphocytes had aggregated in moderate density in the brain tissue surrounding the graft.

In the remaining two grafts breakdown fell just short of completion. In one (Fig. 18) a fragment of epithelium at the graft margin remained basiphilic in staining reaction, and there being some reason (however feeble) for supposing it to be alive, must be judged still living (Medawar, 1944, 1947). In the other (Fig. 19) the graft itself was necrotic, but two or three islands of epidermal epithelium in the tenuous and almost wholly avascular ground substance of the brain adjacent to it were still (by the same token) in a surviving state.

There is other evidence that the breakdown of grafts transplanted to the brains of immunized animals dallies slightly behind the breakdown of their orthotopic controls; for even in the four grafts found to be wholly necrotic at autopsy, the presence of a multilayered cuticle so disposed as to indicate the beginnings of encystment by internal or external closure showed that relatively vigorous mitotic and migratory activity had occurred before the immunity reaction finally overtook them. The cause of this time-lag is not clear. It should be noted, however, that this phase of proliferation and migratory activity overlaps the period during which the invading capillary sprouts may be supposed to have a temporarily increased and therefore abnormal permeability (Abell, 1946). Unless the presence of a skin graft permanently or for very long periods alters the characteristic permeability of brain capillaries in its neighbourhood (which is by no means impossible: Lange, 1944) it can be taken that the graft submits to the immunity reaction after a capillary circulation of not grossly abnormal permeability has been re-established.

DISCUSSION.

The role of vascularization in the homograft reaction.

In rabbit skin homografts the breakdown of dermal blood vessels is very nearly simultaneous with the breakdown of the foreign epithelium; and since some form of vascular stagnation or rupture accompanies the breakdown of skin and other tissue homografts in other common laboratory animals and in man, it has long been thought that the two events are related to each other as cause to effect (Fasiani, 1924).

There are many objections to this view, singly indecisive but together of some weight. For example, homograft epithelium which migrates from the centre of a pinch graft over richly and persistently vascular granulation tissue of native origin breaks down earlier than, if not simultaneously with, the "resident" foreign epithelium of the graft centre (Gibson and Medawar, 1943; Medawar, 1944); and where native and foreign epithelium are in contact, the breakdown reaction distinguishes the two types almost to a cellular boundary (Medawar 1944). It is hard to believe that a process of vascular breakdown could discrimi-

nate between the two so finely. In feebly immunized animals, moreover, the progress of the immune response is indicated by the partial suppression of hyperplasia before the blood vessels of the graft dermis show any perceptible specific abnormality (Medawar, 1946*b*).

The behaviour of homografts transplanted to the anterior chamber of the eyes of specifically immunized rabbits now provides an almost decisive disproof of the hypothesis that ischaemia is the immediate cause of homograft breakdown. For if such grafts are vascularized they are destroyed, and their blood supply is duly arrested; but they do not need to be vascularized in order to remain alive. If the interruption of blood supply were the cause of breakdown, then such a graft should remain alive as a sort of tissue culture *in vivo*, being now on all fours with those eye-implantation homografts that are, in fact, never vascularized at all. They do, however, succumb completely. Vascular breakdown is not, therefore, the primary mode of expression of the immune reaction.

The aqueous humour is variously regarded as a plasma ultrafiltrate or as an active secretory product. In either event it is normally free from protein. The survival of non-vascularized skin homografts in the eye, despite adhesion to the corneal endothelium or very intimate association with proliferating mesenchymal tissue of native origin (see above), and their prompt breakdown after vascularization, suggests that the immune reaction is mediated by blood plasma or by cells transported in it. The systemic nature of the immune reaction (further affirmed by the experiments described in this paper) is consistent with no other belief.

The role of lymphatics.

Homografts transplanted to the brain and (according to one view: see above) the anterior chamber of the eye do not elicit an immune reaction. Homografts transplanted to the brain or which become vascularized in the anterior chambers of animals already specifically immunized do, however, submit to it normally. This disparity of behaviour is consistent with the view that a lymphatic drainage system is required to create a state of immunity but not necessary to enforce a response to it. A similar disparity was recorded by Medawar (1946*b*) in assessing the immunizing power of leucocytes towards skin subsequently grafted from the leucocyte donor. Leucocytes are at least 18 times more effective when injected intradermally than when injected intravenously, i.e. at the point physiologically most remote from lymph nodes in general and from any one set of draining nodes in particular.

Clinical significance: the corneal homograft.

It is now a commonplace of ophthalmic surgical practice that a corneal defect may be repaired by a graft of corneal tissue taken from someone other than its intended recipient. There are at least four possible reasons for the success, or apparent success, of such corneal homografts:

(1) The dosage of foreign tissue involved in corneal homografting may be ineffectively small (Medawar, 1944, 1945). There may indeed (though it has not been demonstrated) be a threshold of dosage below which a homograft cannot immunize its recipient strongly enough to secure its own breakdown. McDonald and Medawar, in unpublished observations, find that mouse skin homografts

differing only slightly in antigenic equipment from their recipients may struggle along and survive despite a manifestly feeble immunization.

(2) Because of the feebleness of immunization, breakdown (if it occurs) may be delayed in onset and prolonged in execution. (Breakdown of the cell population of a rabbit skin homograft may take up to four days from beginning to end ; Medawar, 1944). Since corneal epithelium is highly mobile (Arey and Covode, 1943 ; Mann, 1944), cells of native origin from the periphery of the grafted area may insidiously and imperceptibly replace the foreign epithelium during its protracted breakdown.

(3) Corneal homografts cannot elicit immunity, even if they could succumb to it.

(4) Corneal homografts, being unvascularized, cannot succumb to an immune reaction even if they can initiate one.

The experiments reported on here show that explanation (4) is singly sufficient. If one of the two controversial views outlined in the introduction is accepted, explanation (3) is likewise singly sufficient. If neither (3) nor (4) were true, nothing would prejudice the applicability of explanations (1) and (2) So far from being in any way anomalous, therefore, the observed clinical success of corneal homografts has a stronger theoretical backing than most surgical procedures aspire to.

SUMMARY.

1. Heterotopic grafting has been used as a means for studying the part played by blood and lymph vessels in transplantation immunity.

2. The breakdown of skin homografts transplanted beneath the integument of specifically immunized rabbits is indistinguishable in degree and kind from the breakdown of control grafts transplanted (" orthotopically ") to the skin of the chest.

3. A skin homograft transplanted to the anterior chamber of the eye of a specifically immunized rabbit is destroyed if, and only if, it is penetrated by blood vessels.

4. The fact that vascularized eye implants are destroyed, though they are not dependent on a blood supply for their continued survival, shows that vascular stagnation is not causally connected with the breakdown of skin homografts.

5. Skin homografts transplanted to the brains of specifically immunized rabbits respond by total breakdown to an immune state already in being. On the testimony of other workers, they enjoy prolonged or indefinite survival when grafted to the brains of non-immunized animals.

6. It is concluded that skin homografts transplanted to the brain submit to but cannot elicit an immune state.

7. It is suggested that the presence of a lymphatic drainage system is necessary for immunity to be called into being and that penetration by blood vessels must occur before it can come into effect.

8. The behaviour of heterotopic grafts of all kinds shows that the immune state provoked by skin homografting is systemic in compass and not confined to a reaction in the neighbourhood of the foreign grafts.

9. The clinical success of corneal homografts is explained.

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THE EPIZOOTIC BEHAVIOUR OF MOUSE-POX (INFECTIOUS ECTROMELIA).

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INFECTIOUS ectromelia was first described by Marchal (1930) as a natural virus disease of mice. Following an observation that ectromelia virus produced a haemagglutinin which agglutinated the same restricted group of erythrocytes as did vaccinia virus, Burnet (Burnet, 1945*a*; Burnet and Boake, 1946) established the close immunological relationship between ectromelia and vaccinia, and pointed out that ectromelia was the murine representative of the mammalian pox viruses. Consideration of the symptomatology and epidemiology of the disease led to the suggestion by Professor Burnet (Fenner, 1947*c*) that the disease should be called mouse-pox. I have adopted this name as a synonym for ectromelia when discussing the actual disease in experimental mice, but have retained "ectromelia" to designate the virus itself.

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Greenwood, Hill, Topley and Wilson (1936) studied the experimental epidemiology of mouse-pox by the regular daily addition of normal mice to infected herds. By this means they maintained epizootics for more than three years. They proved that it was possible to maintain the disease indefinitely merely by the constant addition of susceptibles to the herd, and established the great importance of immunization by non-lethal infections in protection of mice of the later cage ages from death. However, the information that these investigators were able to extract from their experiments was limited by the fact that the only available criterion of infection was death.

Burnet's elucidation of the systematic position of ectromelia virus as one of the mammalian pox viruses, the availability of simple and accurate methods of titration of the virus (Burnet and Lush, 1936), and of specific antibody (Burnet and Boake, 1946), made further investigation of the epizootic behaviour of the disease desirable. Experiments were conducted to determine the exact mode of spread (Fenner, 1947 *b* and *c*), and when this had been established the behaviour of two strains of virus was studied by the method of " closed epidemics " introduced by Topley (Greenwood, Hill, Topley and Wilson, 1936). The latter experiments form the basis of this paper, in which a general account will be given of the epizootic behaviour of the disease.

MATERIAL AND METHODS.

The materials and methods used have been described in detail in previous papers (Fenner, 1947*a*).

Two strains of ectromelia virus were used, the " Hampstead " strain of Marchal and the " Moscow " strain, isolated in Professor Soloviev's laboratory and sent to us in 1946 through the kindness of Dr. C. H. Andrewes of the National Institute of Medical Research, England.

Stock virus was prepared from infected chorioallantoic membranes, and virus titrations were carried out on such membranes by the pock-counting method. The preparations of the " Hampstead " strain of virus had undergone about 50 passages on the chorioallantoic membrane, while first egg-passage material of " Moscow " strain was used.

Antibody levels were estimated by the haemagglutinin-inhibition test, the ectromelia antihaemagglutinin (E-AHA) titres being expressed as the reciprocal of the dilution of serum in 1 per cent fowl erythrocyte suspension which would permit partial agglutination of the latter by four haemagglutinating doses of virus.

All mice were bred in the Hall Institute mouse-room and were free from serious enzootic diseases. The stock is heterogenetic, and has been maintained by constant interbreeding between black, white, grey, fawn and brown mice.

Closed epidemics were set up as described by Greenwood, Hill, Topley and Wilson (1936), using a battery of cages similar to those described by Topley (1923). One cage was used for every 20 mice. The mice were changed to clean cages every second day, being examined during the change-over. Cages were sterilized before being cleaned. The cage bottoms were covered with sawdust. Mice were given unlimited tap water to drink, from overhead bottles, and fed with an ample supply of " Barastoc dog cubes." During the winter months the

room was heated, and in both experiments the daily temperature usually varied between 60° F. and 70° F.

A herd of 100 normal male mice had previously been maintained under these conditions for 80 days, one death occurring on the 50th day, due to pneumonia. All the other mice were alive and healthy when the experiment was completed.

EXPERIMENTAL RESULTS.

In earlier experiments (Fenner, 1947*b*, 1947*c*) it was found that the usual mode of entry of the virus was through small abrasions of the skin, with the production of small papules which subsequently ulcerated (primary lesions) at the site of entry. The primary lesion of a mouse exposed to infection with "Hampstead" ectromelia virus is shown in Fig. 1. This was an advanced lesion, having been first seen 5 days earlier. Such lesions healed after about a fortnight and left a deep hairless scar, which persisted for the life of the mouse.

If the infected mouse did not die of the acute disease, with extensive necrosis of the liver and spleen, a skin eruption, the secondary rash, usually appeared on about the 10th day after infection, about 2 days after the appearance of the primary lesion. The lesions of the secondary rash began as small, rather pale thickenings of the skin, and about 2 days after first becoming visible (on the shaved skin) they ulcerated and later became scabbed. Healing occurred under the scabs, and all lesions of the secondary rash had usually healed by the end of the 3rd week, with loss of hair and slight scarring. Fig. 2 shows an extensive scabbed secondary rash.

Maximum infectivity occurred during the early stages of the secondary rash, large amounts of virus being shed from both the primary and secondary skin eruptions, and smaller amounts being excreted irregularly in the faeces, urine and saliva. When the rash healed virus was no longer excreted, and such "convalescent" and recovered mice were not infective.

The Incubation Period of Mouse-pox.

Before describing the epizootics which follow the exposure of herds of normal mice to infected animals, it is necessary to provide data on the incubation period of mouse-pox following natural infection with the two strains of virus, and following the inoculation of known doses of both strains in the foot pad.

The first clinical sign of infection, which marks the end of the incubation period, may be the primary lesion, the secondary rash, or death, and some mice may be infected but show no clinical or even post-mortem evidence except the development of specific antibody.

A number of experiments were carried out in which normal mice were placed in contact with infective mice for 24 hours, then separated and examined daily for evidence of infection. The actual infectivity of the environment varied somewhat in different experiments with the same strain of virus, but the results were sufficiently alike to allow them to be pooled, as has been done in Table I.

In cases in which both a primary lesion and a secondary rash were found, the latter always appeared somewhat later than the former. Death without the appearance of a primary lesion or secondary rash also occurred after a slightly

longer incubation period than preceded the appearance of the primary lesion. The incubation period of mouse-pox has therefore been determined as the interval between exposure to infection and the appearance of the primary lesion, and from Table I it is evident that it is somewhat shorter with the "Moscow" than the "Hampstead" strain of virus (6·8 ± 1·7 days and 8·1 ± 1·4 days). The incubation period of "Moscow" mouse-pox varied between 4 and 10 days, one case (out of 38 with primary lesions) occurring at each of the extremes. With "Hampstead" virus the range was from 6 to 11 days.

Although the degree of virus contamination of the environment was about the same in parallel experiments with the two strains of virus, as judged by the type, age, and severity of lesions in the "infector" mice, the infectivity and the virulence or killing-power of the two strains varied greatly. With the "Moscow" strain only 17 per cent of contact mice escaped infection, but with "Hampstead" virus 70 per cent were not infected. Deaths without the appearance of primary or secondary lesions occurred only with the "Moscow" strain, and inapparent infections only with the "Hampstead" virus.

In six experiments with the "Moscow" strain and four with "Hampstead" virus 3 normal mice were added to the recently exposed animals the day that they were separated from the infective mice, and observed daily for the appearance of primary lesions. As the original contact mice were removed from the tins soon after the first evidence of infection (either the primary lesion or secondary rash) was definitely diagnosed, the risk of infection was low. With the "Moscow" strain, secondary cases occurred amongst about half of these animals at intervals varying from 9 to 16 days after their addition to the tins, the average interval being 12 ± 2 days. With the "Hampstead" strain only one secondary case occurred, 16 days after addition of the contact mice to the tins.

To see whether the longer incubation period of the "Hampstead" strain was due to a smaller initial dose, or to slower multiplication of the virus, groups of mice were inoculated in the pad of the left hind foot with various doses of the two strains, and the first appearance of swelling of the foot determined. The results are shown in Table II.

From this it is evident that with all dosages the incubation period of the "Hampstead" virus is longer than that of the "Moscow" strain. When swabs soaked in various dilutions of virus were rubbed on the lightly scarified dorsum or sole of the hind foot, infection occurred only with suspensions of virus containing at least 10^5 ID_{50} per ml. The incubation period with this dilution was 7·0 ± 1·6 days with the "Moscow" strain and 8·9 ± 1·5 days with "Hampstead" virus.

It is probable, therefore, that the observed differences in the incubation periods of the two strains of naturally contracted mouse-pox are due to differences between the viruses themselves rather than differences in the severity of exposure and average dose of virus received by the contact mice.

EXPLANATION OF PLATE.

FIG. 1.—Advanced primary lesion of mouse-pox, first seen 8 days after exposure of mouse to contact infection with "Hampstead" strain, and photographed 5 days later.

FIG. 2.—The secondary rash of mouse-pox. Widespread scabbed lesions first seen on 10th day after inoculation in the foot with "Moscow" strain, and photographed 4 days later after depilation.

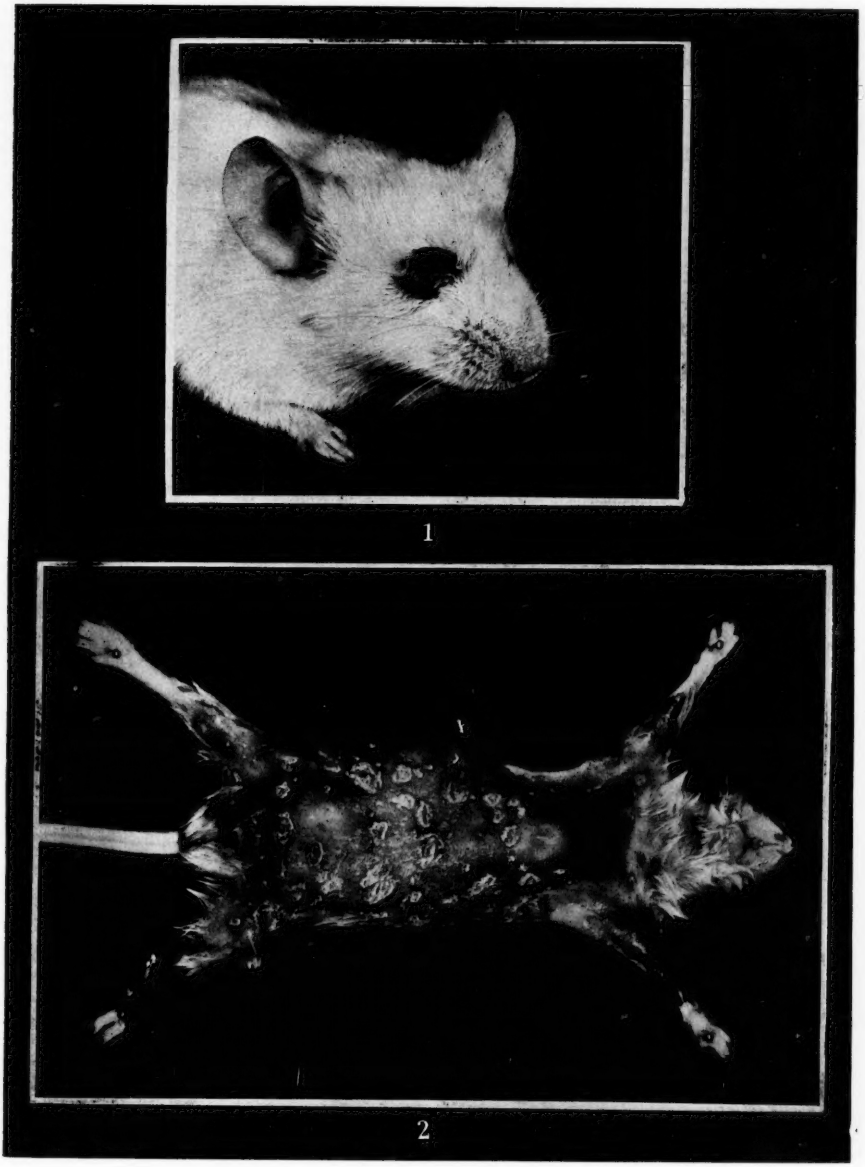

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TABLE I.—*The Incubation Period* of Mouse-Pox, Determined by the Infection of Normal Mice by Exposure to Infective Mice for 24 hours.*

Strain of virus.	Number of mice.	Percentage of mice showing as first evidence of infection: Not infected.	Primary lesions: Percentage.	Primary lesions: Incubation period.	Secondary rash: Percentage.	Secondary rash: Incubation period.	Deaths: Percentage.	Deaths: Incubation period.	Serological evidence only.
"Moscow"	60	17%	63%	6·8 ± 1·7 days.	7%	11 days	13%	8·4 ± 1·4 days	0
"Hampstead"	91	70%	17%	8·1 ± 1·4 „	4%	10 „	0	...	9%

* Incubation period expressed as the arithmetic mean ± standard deviation of the number of days elapsing between the day of exposure (day 0) and the first evidence of infection.

TABLE II.—*The Incubation Period* of Mouse-Pox Determined by the Inoculation of 0·05 ml. of Various Dilutions of Ectromelia Virus into the Pad of the Hind Foot, or by Rubbing a Swab Soaked in Virus-Containing Fluid on the Scarified Foot.*

Strain of virus.	Pad inoculation: Dose of virus. †	Pad inoculation: Number of mice.	Pad inoculation: Incubation period.	Scarification: Dose of virus. ‡	Scarification: Number of mice.	Scarification: Incubation period.
"Moscow"	5	9	10 days	...	...	...
	5×10^2	23	7·2 ± 1·2 days	5×10^6	14	7·0 ± 1·1 days
	5×10^7	10	3·9 ± 0·3 „	5×10^9	16	4·0 ± 0·5 „
"Hampstead"	5	10	11·4 ± 0·8 „	...	...	...
	5×10^2	24	8·4 ± 0·7 „	5×10^6	9	8·9 ± 1·5 „
	5×10^7	10	4·9 ± 0·7 „	5×10^9	16	5·7 ± 0·8 „

* Incubation period expressed as arithmetic mean ± standard deviation of the number of days between inoculation (day 0) and the first detectable swelling of the foot.

† Dose expressed as approximate number of ID_{50}, determined by pad inoculation of mice, contained in 0·05 ml. of inoculum.

‡ Dose expressed as approximate number of ID_{50}, per ml. of suspension in which swab was soaked.

Closed Epidemics of Mouse-pox.

The course of the disease in closed epidemics in herds of a hundred mice was studied. To about 100 10–11 weeks old male mice, housed in six cages in series, were added 15 mice which had been inoculated in the pad of the left hind foot with 1 : 100 dilution of ectromelia virus on the previous day. The courses of the epidemics which followed are shown in Fig. 3 and 5, which demonstrate respectively the secular trend in mortality and morbidity.

Post-mortem examinations of fatal cases were carried out as soon after death as possible, to obviate the risk of cannibalism. This factor did not complicate these experiments, as in only one mouse, that was killed by its cage-mates on the third day of experiment CIII, was a considerable part of the corpse eaten. In experiment CIV small parts of the heads of 4 dead mice were eaten.

All contact mice were carefully examined for evidence of skin lesions every second day from the 10th day after addition of the inoculated mice until the end of the experiment.

Experiment CIII:" Moscow " Ectromelia Virus.

In this experiment the " Moscow " strain of ectromelia virus, which has been found to be considerably more virulent after inoculation than the " Hampstead " strain, was allowed to spread among 97 normal 10-weeks-old male mice. All the inoculated mice had swollen left hind feet by the 5th day, and between the 5th and the 7th days 12 of the 15 had died. The three survivors developed secondary rashes and conjunctivitis on the 9th day and these lesions became widespread and severe during the next week. They cleared up during the 3rd week and were quite healed by the 21st day.

On the 13th day a wave of mortality commenced in the contact mice, and by the 24th day 50 mice had died from acute mouse-pox. By this time all but 4 of the survivors had developed a secondary rash and only one of these died subsequently, on the 33rd day. On that day the experiment was concluded by sacrifice of the remaining animals. During post-mortem examinations blood was collected for antibody estimations.

The primary lesion.

Primary lesions were found in 72 of the 97 contact mice, and as Table III indicates, the majority of the cases in which primary lesions were not detected were fatal. These mice presumably died before local multiplication of the virus at the site of entry was sufficient for an obvious lesion to develop. Two primary lesions were found in each of three fatal cases. Table III shows the distribution of primary lesions, two entries being made when there were two primary lesions.

It can be seen that the primary lesions usually develop on areas of skin in constant contact with the environment. The rarity of primary lesions of the feet has been noted before (Fenner, 1947*b*), and is presumably due to the thickness of the skin of the pads of the feet. The lesions noted in Table III as occurring

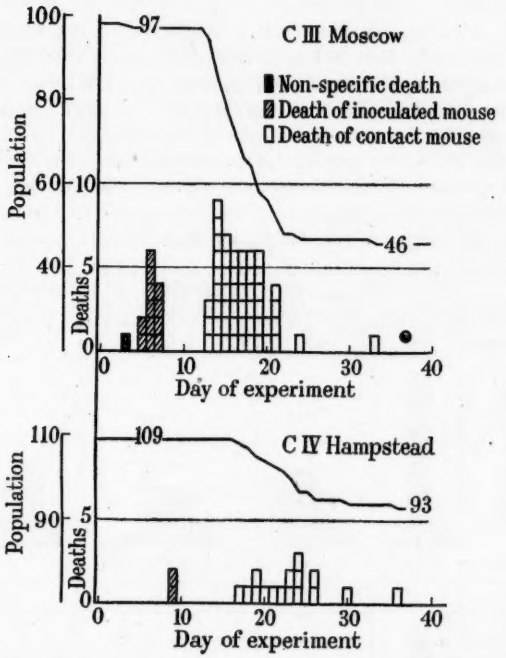

FIG. 3.—Mortality in epidemics of "Moscow" and "Hampstead" mouse-pox among normal adult male mice. Each block indicates the death of one mouse.

on the feet involved the hairy skin of the dorsum of the foot rather than the pad itself.

TABLE III.—*Distribution of Primary Lesions in Epidemics of Mouse-Pox.*

Site.	Experiment CIII, "Moscow" strain.		Experiment CIV, "Hampstead" strain.	
	Fatal cases.	Survivors.	Fatal cases.	Survivors.
None found	19	6	6	22
Head	6	8	4	23
Nose	6	12	3	20
Eye	4	7	2	11
Lower lip	3	4	0	3
Belly	13	6	2	4
Fore foot	2	3	0	7
Hind foot	1	2	0	1
Neck	0	0	0	5
Tail	0	1	0	0
Totals	54	49	17	96

The secondary rash.

The secondary rash was first detected by feeling small lumps in the skin of the back when it was picked up between the index finger and the thumb. Study of the rash in shaved mice, in which the earliest appearance of skin lesions could be detected, showed that nodular thickening of the skin of the back could be felt within 24 hours of the first appearance of the rash.

In only 4 of the 46 survivors did no secondary rash develop. Only 3 of the 45 mice which developed a secondary rash died subsequently, 1 on the 21st, 1 on the 22nd, and 1 on the 33rd day of the experiment. In the other 42 mice the rash varied in severity and duration, as shown in Fig. 4.

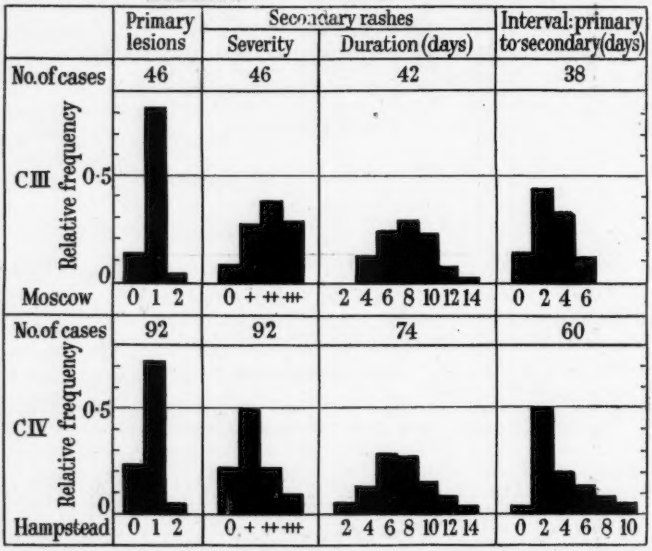

FIG. 4.—The occurrence, severity and duration of primary lesions and secondary rashes in survivors of epidemics of "Moscow" and "Hampstead" mouse-pox, and the interval between the occurrence of such skin lesions. Primary lesions: 0, 1, 2 = number of primary lesions found. Secondary rashes: 0, + etc. = varying degree of severity.

Most of the "slight" rashes healed in 4 or 5 days, the "moderate" ones usually within 8 days and the "severe" in 8 or 10 days. One mouse had a severe secondary rash for more than 15 days. The curve showing the daily frequency of "new rashes" was a unimodal curve of lower amplitude than the morbidity rate curve (because many mice died), and centred about 3 days later than the latter curve (Fig. 5).

The morbidity rate.

Fig. 3, which is the orthodox type of diagram used for the analysis of experimental epidemics, shows the daily deaths, but takes no account of the time of infection of those mice which were infected but survived. By means of the

primary and secondary skin lesions it was possible to construct another curve, shown in Fig. 5, which illustrates the number of mice which showed the first definite evidence of infection each day. In order to make the greatest possible use of the available data the following criteria of infection were adopted: (1) the first appearance of a primary lesion, (2) in acute fatal cases with no primary lesion, the day of death, and (3) in survivors in which a secondary rash developed, but no primary had been detected, a deduction of 3 days was made from the

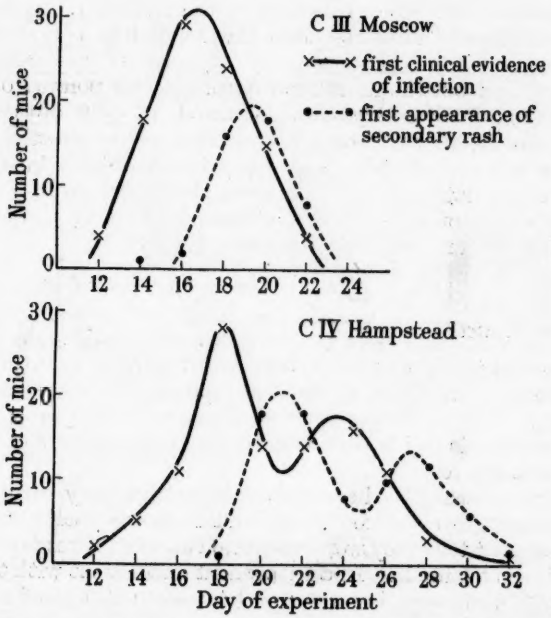

FIG. 5.—Morbidity curves of epidemics of " Moscow " and " Hampstead " mouse-pox among normal adult male mice. Morbidity = the first clinical evidence of infection, either the primary lesion, death from the acute disease, or with a deduction of 3 days, the first sign of the secondary rash (see text). The broken curves show the number of mice in which secondary rashes were seen for the first time on the day indicated.

first day on which the secondary was seen, this being the average interval between the detection of primary and secondary skin lesions.

With " Moscow " virus this curve showing the secular trend of morbidity is almost symmetrical with the mode on the 17th day.

Mice dying of acute mouse-pox do not shed much virus into the environment. For instance, in 12 experiments in which 2 normal mice were included as contacts with groups of 4 inoculated mice, only 2 of the 12 contacts were infected in six cages in which all the inoculated mice died before the 7th day, while 11 of 12 mice were infected in the six cages in which at least one mouse survived for 10 days or longer.

In this experiment ulceration of the feet occurred in only 5 of the 12 acute fatal cases, and it is unlikely that these mice, dying on the 5th, 6th and 7th days of the experiment, shed much virus into the environment. However, as ulceration of the feet was evident in two mice on the 5th day, that day must be regarded as the first on which the contact mice were exposed to infection. The period of maximum infectivity was probably between the 9th and 12th days. The first contact cages showed evidence of infection on the 12th day, and the largest number showed lesions on the 16th day. All contact mice showed clinical evidence of infection by the 22nd day.

Previous experiments indicated that the incubation period of the natural disease was about 7 days. The majority of cases in this epizootic probably contracted infection from the inoculated mice. It was pointed out earlier that in other experiments secondary cases occurred 12 ± 2 days after primary infections, the shortest interval being 9 days. Secondary infections might therefore account for a few of the late cases, but with this highly infectious strain of virus the overlap of late primary and early secondary cases is complete, and both mortality and morbidity curves indicate an explosive epidemic with no clearly separated primary and secondary waves.

Post-mortem appearances.

Fatal cases.—Forty-six of the 51 fatal cases showed acute lesions at post-mortem, consisting of necrosis of the liver and spleen, and enlarged engorged superficial lymph nodes. Twenty-one of these mice had early conjunctivitis. Seven had haemorrhagic foci in both kidneys, and the lungs of 2 of these 7 showed multiple haemorrhagic foci.

In a few of these acute fatal cases there were a few very early lesions of the secondary rash and they occurred in the middle of the back. It was not until late in the experiment that they were recognized and no figures for their frequency in this epidemic are available. In a subsequent experiment with the same strain of virus, however, such early lesions were detected in 10 out of 27 acutely fatal cases. In 2 mice dying of the acute disease, small haemorrhagic spots were scattered throughout the skin and subcutaneous tissue.

Two of the other 5 fatal cases had more advanced necrotic lesions than the others, having lived for a few days longer after being infected. The remaining 3, which died 6, 10 and 17 days after the appearance of the primary lesion, had extensive scabbed secondary rashes. They were thin animals, their livers were small but not necrotic and their spleens were small and pale with no necrotic lesions.

Survivors.—The 46 survivors were killed on the 33rd day, when the rashes of all of them had completely healed. The post-mortem appearances varied somewhat. The superficial lymph nodes were usually enlarged, except in the few cases in which no secondary rash occurred. Fibrotic lesions of the spleen occurred in 29 cases. The liver was usually somewhat enlarged, but macroscopic necrotic foci did not occur. Microscopically such livers showed focal collections of endothelioid and small round cells around many branches of the portal tract. The cytoplasm of the liver cells showed considerable variation in intensity of

staining, their nuclei varied in size, shape, and intensity of staining, and diffuse fatty changes were present.

The sites of the primary lesions, and of individual lesions of the secondary rash, were usually evident as hairless scars.

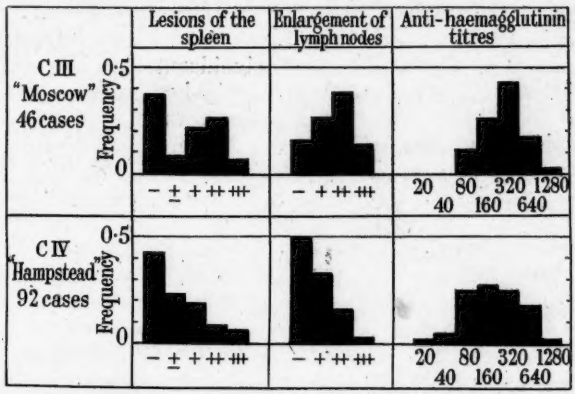

FIG. 6.—Post-mortem lesions and antibody titres in survivors of epidemics of "Moscow" and "Hampstead" mouse-pox. 0, ±, + etc. indicate varying degrees of severity of lesions of the spleen and the superficial lymph nodes. ("±," referring to the spleen, indicates the presence of a few minute white plaques on the surface.)

The serological response in survivors.

E-AHA titres were determined on all sera collected, and in this experiment, titres varied between ·80 and 1280 (Fig. 6). The geometric mean titre was $260 \times\!\div 1{\cdot}9$. Scatter diagrams were constructed and the correlation coefficients determined between antibody levels and the severity of secondary rashes, of lesions of the spleen, and the degree of enlargement of the superficial lymph nodes. The correlation coefficients are shown in Table IV. Tests for the significance of the correlation coefficients are taken from Fisher (1944). The enlargement of the superficial lymph nodes was associated with the secondary rash, but varied according to the time which had elapsed between healing of the rash and the post-mortem examination. It has therefore been omitted from subsequent comparisons which are based on the E-AHA titre, the severity of internal lesions, as shown by the degree of fibrosis and scarring of the spleen, and the severity of the secondary rash.

In this experiment no significant correlation was found between the severity of the internal and the external lesions. This is probably due to the small number of mice in the series (46), for when the post-mortem findings of 31 survivors amongst 100 normal mice exposed to "Moscow" ectromelia virus in another experiment are added to the results of this experiment, a significant correlation (correlation coefficient 0·58) is found. That is, if an animal which survives the acute attack later develops a severe secondary rash, it is likely that it will also show at post-mortem severe internal lesions.

TABLE IV.—*Correlation Coefficients of Antibody (E-AHA) Titres, the Severity of the Secondary Rash, and the Degree of Fibrosis of the Spleen.*

Comparison of severity of—	CIII, "Moscow" strain. Correlation coefficient.	CIII, "Moscow" strain. Significance.	CIV, "Hampstead" strain. Correlation coefficient.	CIV, "Hampstead" strain. Significance.
Fibrosis of spleen and secondary rash	·22	Not significant	·61	Highly significant
	·58*	Highly significant		
Fibrosis of spleen and E-AHA titre	·43	Ditto	·68	Ditto
Secondary rash and E-AHA titre	·39	,,	·50	,,
Fibrosis of spleen plus secondary rash and E-AHA titres	·42	,,	·79	,,

* This figure was derived from the pooled results of Experiment CIII and another experiment involving the spread of "Moscow" ectromelia virus among normal mice.

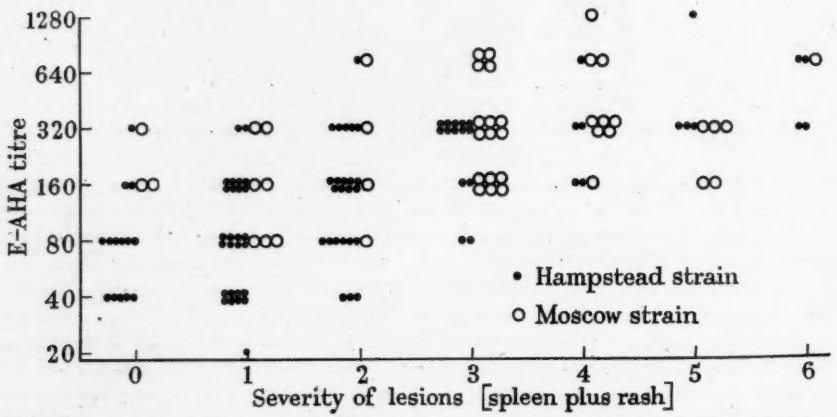

FIG. 7.—Scatter diagram showing the correlation between the severity of lesions in survivors of experiment CIII ("Moscow" strain) and CIV ("Hampstead" strain) and the antihaem-agglutinin titres. Correlation coefficients 0·42 and 0·79 respectively. The severity of spleen lesions and of the rash were each allotted a score of 0, 1, 2, or 3 and added. For each mouse this total score was plotted against the E-AHA titre.

There was a significant correlation between the severity of each type of lesion and the height of the E-AHA titre. When the severity of external and internal lesions was combined, by allotting a score of 0, 1, 2, 3, to the varying degrees of severity of both spleen lesions and secondary rash and adding them, and then plotted against the E-AHA titres of the animals concerned, a high degree of correlation is evident (Fig. 7).

It is evident, therefore, that the height of the antihaemagglutinin response is determined largely by the intensity and duration of the antigenic stimulus, which is indicated by the severity of lesions of the skin and of the spleen.

Experiment CIV : "Hampstead" Ectromelia Virus.

One hundred and nine 11-weeks-old male mice were exposed to infection by 15 mice which had received 0·05 ml. of 1 : 100 "Hampstead" ectromelia virus in the pad of the left hind foot the day before. The course of this experiment, in terms of mortality, is shown in Fig. 3.

The feet of the inoculated mice were swollen on the 5th day, and scabbing was first evident on the 6th day. Two mice died on the 9th day. After reaching a maximum degree of swelling with scabbing on the 10th day, the lesions of the feet started to regress on the 12th day. A secondary rash developed in 5 of the 13 survivors, but it became severe and widespread in only one. All lesions of the inoculated mice had healed by the 20th day.

Amongst the contact mice there was no sharp wave of mortality such as occurred in the previous experiment. Instead there were only 16 deaths, 14 of which occurred between the 17th and 26th days. By the 32nd day all but 4 of the mice had shown some evidence of infection. The experiment was concluded on the 36th and 38th days, when all survivors had recovered from the effects of the disease.

The primary lesion.

Primary lesions were found in 81 of the 109 contact mice (Table III), the proportions of mice in which primary lesions were not detected being about the same in the 2 epidemics.

The majority of primary lesions again occurred on and around the head. Lesions of the belly wall were less frequently detected than in the previous epidemic.

The secondary rash.

As can be seen from Fig. 4, the secondary rashes were distinctly less severe, and lasted for shorter periods in this epidemic than in the previous experiment. In 18 mice secondary rashes constituted the only clinical evidence of infection. Four animals developed no external lesions whatever throughout the experiment, although serological examination indicated that they had been infected.

The curve showing the daily frequency of "new rashes" closely parallels the daily morbidity curve, showing, like the latter, a major and a lesser peak. The interval between corresponding points on the two curves is about 3 days (Fig. 5).

The morbidity rate.

For experiment CIV this curve (Fig. 5) was calculated on the same basis as before. It differs greatly from that found previously, for there are two waves, a larger one with the mode on the 18th day, and a smaller one with the mode on the 24th day.

The incubation period of " Hampstead " mouse-pox is about 8 days, and the experiments described earlier showed that it was much less infectious than the " Moscow " strain. The inoculated mice were first infective on the 6th day, and maximum infectivity probably occurred on the 9th to 12th days. The build-up of the primary wave was considerably slower than with the " Moscow " strain, and the peak occurred on the 18th day. This primary wave, accounting for 60 per cent of the infected mice, was complete on the 20th day. After a short interval it was followed by a rather broad secondary wave of cases infected from the earlier cases of the primary wave.

Post-mortem appearances.

Fatal cases.—The post-mortem appearances in the acute fatal cases were similar to those described in experiment CIII. Lesions of the kidneys occurred in 4 cases. One case showed an early secondary rash at the time of death, and the liver and spleen of this mouse showed more advanced necrotic changes than are commonly seen. One mouse with a severe secondary rash died after being sick for several days. It had severe bilateral conjunctivitis, the primary sore on the nose showed no sign of healing, although it had been present for 16 days, and the skin was covered with crusted lesions. The lymph nodes were greatly enlarged, and the spleen showed extensive advanced necrotic lesions.

Survivors.—The lesions of the lymph nodes and of the spleen were on the whole much less severe than in experiment CIII, and in many mice the only post-mortem evidence of infection was the occurrence of a few, often only one or two, small raised white plaques about 1 mm. in diameter on the ventral surface of the spleen. Microscopically these were seen to be due to localized areas of proliferation of the serosal cells of the splenic capsule. In mice in which the secondary rash had been severe there were many small hairless areas where scabs had separated, and in many mice the site of the primary lesion was marked by a deep hairless scar.

Fig. 6 shows the relative frequency of lesions of the spleen and lymph nodes of different degrees of severity, and shows that as with mortality rates and the severity of the secondary rash, infection with the " Hampstead " strain caused less severe lesions than did infection with " Moscow " virus.

Mention was made before of 4 mice in which there was no clinical evidence of infection throughout the experiment. At post-mortem 2 of these mice were quite normal, but had E-AHA titres of 80 and 40 respectively, and in the other 2 there were minimal lesions of the spleen, consisting of a single small raised plaque on the ventral surface of the organ. The E-AHA titres of these 2 animals were also 80 and 40. Thus in this experiment in which " Hampstead " ectromelia virus spread through a herd of normal mice infections of all degrees of severity, occurred, ranging from death with acute necrosis of the liver and spleen, through survival with skin rashes of varying severity to completely subclinical infections, in which there was a specific antibody rise but no other change.

The serological response in survivors.

The E-AHA titres were on the whole lower than in the more severe " Moscow " infection (geometric means 140 $\times\div$ 2·4 and 260 $\times\div$ 1·9), although individual mice

which suffered severe attacks of mouse-pox developed titres as high as any found in the previous experiment.

Study of scatter diagrams (Fig. 7) and the determination of correlation coefficients (Table IV) indicated that, as before, the height to which the E-AHA titre rose was closely correlated with the duration and intensity of the antigenic stimulus as shown by the severity of lesions of the skin and spleen.

Mouse-pox in Young Animals.

The experimental epidemics just described indicate that among adult mice, under conditions of severe and continuous exposure, mouse-pox is a highly infectious disease with a mortality rate which varies with the strain of virus used, being about 50 per cent with the more virulent " Moscow " strain and 15 per cent with the " Hampstead " strain.

Nursling mice, if infected before the 14th day of life, invariably die after inoculation with small doses of either strain of virus (unpublished observations). The few available accounts of the enzootic disease in laboratory colonies (Marchal, 1930 ; McGaughey and Whitehead, 1933 ; Kikuth and Gönnert, 1940) do not mention a high mortality amongst suckling mice, although apparently the majority of animals are infected by the time they reach adult life. There are probably two mechanisms which protect young animals during the period of suckling, when the case-mortality rate is so high. Firstly, chronic and convalescent infective virus " carriers " do not occur or are very rare (Fenner, 1947*c*), and the chance of a nursing mother actually suffering from the disease, and therefore being infective, is small. It is the usual procedure to separate breeding females from the rest of the stock, and the young are thus sheltered from infection. Secondly, protective antibody might be passed from mother to young, and be available to protect the young against such infection as might occur. The first of these possibilities cannot be studied experimentally, but the development of a method of measuring the E-AHA titre with small volumes of serum (Fenner, 1947*a*) made possible an investigation of the passage of antibody from mother to young.

Passage of maternal antibody to young mice.

Ehrlich (1892), in his classical studies on the passage of immunity against abrin and ricin from adult to young mice, found that the main factor responsible for protection of young mice was the passage of antibody in the milk. Whenever careful investigations have been made with mice, Ehrlich's results have been confirmed (e.g. Culbertson, 1940, Berry and Slavin, 1943). Culbertson (1938, 1939) also found that in rats immunity to *Trypanosoma lewisi* and *T. cruzi* was transmitted in the milk rather than transplacentally. It appears that mice and probably rats are exceptions to the generalizations of Ratner, Jackson and Gruehl (1927) and Needham (1931) that in rodents, which have hemichorial placentas, maternal antibody is passed to the young almost entirely through the placenta.

The results with mouse-pox are in essential agreement with those of Ehrlich and Culbertson, but they give somewhat greater precision to the quantitative relationships between antibody in the mother, the foetus, and the suckling mouse,

and they illustrate the importance of circulating antibody in protection against mouse-pox.

Using normal and immune mothers with newborn young, half of each litter was exchanged so that suckling mice of 4 categories were available, viz. offspring of normal mothers suckled by normal mothers, offspring of normal mothers suckled by immune mothers, offspring of immune mothers suckled by immune mothers, and offspring of immune mothers suckled by normal mothers. At various intervals young mice from each group were killed and blood collected in capillary tubes. E-AHA titres were determined on separate litter-mates for the first 3 weeks, and thereafter the same young mice were bled from the tail at weekly intervals. The results of several experiments were essentially the same as those shown in Fig. 8. They confirm Ehrlich's contention that in mice passage of antibody in the milk is the principal mode of transmission of passive immunity from mother to young.

It was impossible consistently to obtain newborn mice which had not suckled at all, and there was therefore some doubt as to whether the antibody found in the serum of newborn young was acquired through the placenta or in the milk or colostrum. This was solved by killing a pregnant immune female just before term, and determining the E-AHA titres of the sera of the mother and of the 6 embryos removed from the uterus. The E-AHA titre of the mother's serum was 200 and of the embryos 20, confirming the results found in previous experiments (Fig. 8). Thus in mice antibody passes through the placenta to the foetus,

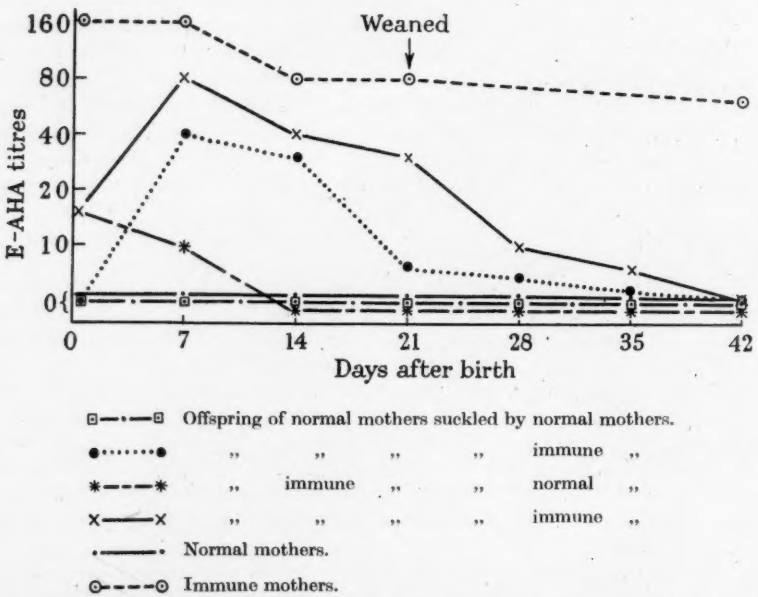

FIG. 8.—Ectromelia antihaemagglutinin titres in the offspring of normal and immune mothers at various intervals between birth and the sixth week.

but only reaches a titre one-tenth as high as that of the mother. This is reminiscent of Wiener and Silverman's (1940) results on the permeability of the human placenta to such antibodies as the syphilitic reagin, isoagglutinins, and certain heteroagglutinins. In humans, however, most ordinary antibodies, such as those developed in response to bacterial or virus infections, pass readily through the placenta, and reach approximately the same titre in cord blood as in the mother's serum (Smith, 1946). In the mouse this antibody was fairly quickly lost, however, and unless the young animal suckled an immune mother all antibody had disappeared by the 14th day. After suckling an immune mother for a few days the antibody titre of the young, whether borne by a normal or an immune mother, rose to a level half as great as that of the nursing mother. After weaning on the 21st day the antibody titres of all young which suckled the immune mother fell gradually until no antibody was detectable at the end of the 6th week.

The question arises as to whether this antibody occurs in the colostrum or the milk, and whether it can be absorbed by mice a few days old as well as by the newborn. I have been unable to obtain any information on colostrum in mice, but several experiments showed that antibody was present in the milk up to 2 weeks after birth of the young, and that such antibody could be absorbed by mice at least 7 days old. If, for instance, normal 7-day-old mice were suckled by an immune mother with an E-AHA titre of 40, the E-AHA titres of these animals on the 14th day was 16, compared with 20 for their litter-mates born of the same normal mother and suckled by the immune mother from birth.

On several occasions milk was removed from the stomachs of young mice, centrifuged, and the supernatant tested for E-AHA. Only one test was successful, and the titre in this case was the same (80) in the mother's serum and the milk.

It will be remembered that the Rockefeller Institute workers (Smadel and Hoagland, 1942) were unable to define the antigen responsible for the development of protective antibodies to vaccinia virus. No investigations have been carried out on the antigens of ectromelia virus, and there is no evidence that the antihaemagglutinin is the protective antibody. Earlier experiments (Fenner, 1947*a*) indicated that there was a close but not absolute correlation between antihaemgglutinin titre and protection against intraperitoneal inoculation of ectromelia virus. To see how well the variations in antihaemagglutinin titre just described indicated variations in immunity of the young, cross-transfers of the young of 4 pairs of normal and immune mothers were made at birth. Fourteen days later the young in the various litters were inoculated in the pad of the hind foot with various dilutions of "Moscow" ectromelia virus. The results are recorded in Table V, and show that the E-AHA titres estimated earlier give an accurate measure of protective antibody, and further that while circulating antibody had little effect on the development of the local lesion, it had a very considerable effect in preventing generalization of infection and death of the mice.

Antibody acquired through the placenta had no apparent effect on the course of the disease, but that acquired by suckling prevented death except when at least 1000 mouse-infective doses were inoculated. It is probable that the single mouse suckled by a normal mother which survived inoculation of a 10^{-7} dose of virus was not infected, for no antihaemagglutinin was present 14 days later.

TABLE V.—*Fate of Mice Born by Normal and Mouse-Pox Immune Mothers, Distributed at Birth among Normal and Immune Mothers, and Inoculated 14 days later in the Pad of the Hind Foot with various Dilutions of "Moscow" Ectromelia. (Chorioallantoic titre* 1×10^8 *Infective Particles per ml.)*

Dilution of "Moscow" ectromelia virus.	E-AHA titre of immune mothers.	Suckled by normal mother.		Suckled by immune mother.	
		Offspring of normal mother.	Offspring of immune mother.	Offspring of normal mother.	Offspring of immune mother.
10^{-7}	40	8, 8, — (0)	10, 11	— (20), — (20)	A, — (40)
10^{-6}	60	8, 12	7, 7, 8	A, A	A*, ++, — (20)
10^{-5}	80	7, 7, 7	7, 8	A, A, +++	A, A, +++
10^{-4}	80	7, 8, 11	7, 7, 8	7, 7, 8	7, 8, A

7, 8, etc. = Death from acute mouse-pox on 7th, 8th, etc., day after inoculation.
—(20) etc. = No local or general lesions at post-mortem, E-AHA titre = 20 etc.
A = Gangrene of inoculated foot followed by amputation.
++, +++, = Moderate and severe lesions of the inoculated foot, short of amputation.

* This was the only mouse suckled by an immune mother which survived the inoculations, but developed lesions of the liver and spleen.

The survivors without lesions of the feet, among mice suckled by immune mothers, had antibody titres somewhat higher than would be expected from passive transfer alone, and therefore probably received an infective dose of virus. Intraperitoneal inoculation of varying dilutions of "Hampstead" virus in 23-day-old mice suckled by normal and immune mothers gave results similar to those shown in Table V, almost complete protection from death being conferred on young suckled by immune mothers against doses 1000 times larger than killed all young suckled by normal mothers.

Mouse-pox in Wild Mice.

The only mention of the discovery of wild mice infected with ectromelia virus is that by McGaughey and Whitehead (1933), who found a few wild mice which had died of this infection in a laboratory in which the laboratory mice were infected. A small survey was made of wild mice from various parts of Victoria. Batches, each of about 10 wild mice (*Mus musculus*) were caught in various parts of the State and forwarded to the laboratory. Here they were killed, their pooled serum tested for E-AHA, and suspensions of pooled ground spleens inoculated intracerebrally in normal mice. A total of 13 batches was thus investigated, but on no occasion was evidence of past or present infection with mouse-pox found. The persistence of ectromelia anti-haemagglutinin (titres of 20–40 being found for at least 6 months after infection), and the specificity and simplicity of the test, make serological investigation for mouse-pox a useful adjunct to any investigations of diseases of wild mice. Such studies in a country where mouse-pox was enzootic would, as Burnet (1945*b*) has pointed out, throw considerable light on the natural history of the mammalian pox diseases.

DISCUSSION.

The experimental epidemics show that the conclusions drawn previously (Fenner, 1947*b*) on the mode of infection with mouse-pox were essentially correct, and that infection occurs through small abrasions of the skin, with the production of a local lesion at the site of entry of the virus.

The extensive skin eruption seen in mice which survive the acute infection appears at the same time, in relationship to time of infection and the onset of symptoms, as does the rash of small-pox, and this rash brings mouse-pox strikingly into line with the latter disease. Observations on the pathogenesis of the rash and its relationship to the distribution of virus and the development of antibody are being carried out.

In most previous studies on experimental epidemiology little attempt has been made to record and assess the clinical features of infection, other than the fact of death, in individual animals. Our experience with the present investigation has shown that the possession of definite criteria of infection in addition to death, such as the primary and secondary lesions, which occur at defined intervals after infection, plus the serological findings, adds greatly to the information which can be gained from the method. Comparison of the information available from a study of Fig. 3 and 5 adequately illustrates this.

There is a good deal of evidence that strains of ectromelia virus of varying virulence occur. Schoen (1938) found that when tested under comparable conditions by intraplantar inoculation, the virus which she isolated in Paris was more virulent than the " Hampstead " strain. Similarly all the experiments performed in this laboratory indicate that the " Moscow " strain is of considerably higher virulence than the " Hampstead " strain.

TABLE VI.—*50 per cent Infective and 50 per cent Lethal Doses of Two Strains of Ectromelia Virus Inoculated Intraperitoneally and into the Pad of the Hind Foot. (50 per cent Chorioallantoic Infective Doses of All Preparations about the same, viz.* 10^{-7}.)

Strain of virus.	Natural spread. Percentage mortality.	Pad inoculation.		Intraperitoneal inoculation.	
		ID_{50}.	LD_{50}.	ID_{50}.	LD_{50}.
" Moscow "	53%	$10^{-8.0}$	$10^{-6.5}$	$10^{-8.0}$	$10^{-7.7}$
" Hampstead "	15%	$10^{-8.0}$	$10^{-1.4}$	$10^{-8.0}$	$10^{-4.8}$

In Table VI the ID_{50} and LD_{50} (estimated by the method of Reed and Muench, 1938) of the two strains of virus, inoculated intraperitoneally and in the pad of the foot, are set out. With these two strains of virus the following differences are evident :

(1) With material of substantially the same chorioallantoic titre the ID_{50}'s of both strains by both routes of inoculation are much the same, but the LD_{50} of the " Moscow " strain is about 1000 times higher than that of the " Hampstead " strain after intraperitoneal inoculation and 100,000 times higher after pad inoculation.

(2) After exposure to much the same amount of virus in a contaminated environment for a period of 24 hours, nearly 3 times as many mice were infected when the " Moscow " strain was used than with the " Hampstead.. " strain, and the incubation period of infections due to the latter strain of virus was about a day longer (Table I).

(3) When allowed to spread through herds of about 100 mice the " Moscow " strain caused an explosive outbreak in which all mice were infected by about the

10th day after the inoculated mice first shed virus into the environment, while the " Hampstead " strain caused a primary wave of cases comprising 60 per cent only of the total herd, the remaining 40 per cent being secondary cases.

(4) Under conditions of natural spread the mortality rate was 3½ times greater with the " Moscow " strain than with the " Hampstead " virus, and among the survivors severe necrosis and fibrosis of the spleen, severe secondary rashes and high antibody titres were much more frequent with the " Moscow " strain. Clinically inapparent infections occurred only with the " Hampstead " strain.

The nature and cause of these differences will not be discussed here, as they are the subject of further study, but the analogy of these two strains of mouse-pox to variola major and alastrim is interesting.

Ectromelia virus has been isolated from laboratory mice in England (Marchal, 1930 ; McGaughey and Whitehead, 1933 ; Fairbrother and Hoyle, 1937 ; Andrewes, 1939), France (Schoen, 1938), Germany (Kikuth and Gönnert, 1940), and Russia (Andrewes, 1946—personal communication). No account has been published of the occurrence of mouse-pox in laboratory stocks in America, but Dr. L. C. Strong (personal communication) states that ectromelia has occurred sporadically in his mouse colony several times during the last 28 years. It was recognized as a disease of young mice with a rather low mortality rate, and was followed not infrequently by amputation of the tail or the hind foot.

Infection usually became apparent in one of two ways: Sometimes (Marchal, 1930 ; McGaughey and Whitehead, 1933 ; Schoen, 1938) a high mortality amongst mice assembled after weaning or after young mice had been received from outside breeding establishments led to an investigation of the cause of death, which proved to be mouse-pox. At other times (Fairbrother and Hoyle, 1937 ; Hornus and Thibault, 1939 ; Kikuth and Gönnert, 1940) ectromelia virus was isolated after serial passage of various organs (lungs, spleen, liver) of mice during the investigation of other diseases. This situation, marked by the occurrence of either disastrous epizootics or trivial unnoticed infection, resembles the conditions obtaining with sheep-pox in sheep (Burnet, 1945*b*), and virus diseases like infectious hepatitis or small-pox in man (where vaccination does not modify the picture). The acute widespread epizootics or epidemics marked perhaps by a high mortality represent the introduction of strains of virus into a non-immune population. Either initially or perhaps as a result of free passage an unusually virulent strain may be concerned in such episodes. Where the disease is enzootic or endemic the general rule is that strains are of relatively low virulence, causing chiefly low-grade or inapparent infections amongst the young with a low mortality rate and a high proportion of immune individuals in the population.

The only account available of the epizootic behaviour of mouse-pox is that of Kikuth and Gönnert (1940). After finding that influenza virus maintained in their laboratories became replaced by ectromelia virus during serial mouse-lung passage, they investigated 5 separately maintained stocks of laboratory mice by serial intranasal or intraplantar passage of lungs or feet of apparently normal mice. They found that mice of 2 of the 5 strains were infected with ectromelia virus, which was isolated on a single passage of both lungs and feet in one case, and on the third lung-passage in the other. They interpreted this as indicating that mouse-pox infection was latent in these two stocks and could

be " activated " by any type of non-specific stimulus, comparing the fluctuating host-parasite equilibrium with that of herpes simplex in man.

An attempt was made to repeat Kikuth and Gönnert's experiments using 24 mice which had recovered from infections with " Moscow " and " Hampstead " strains of virus contracted at various periods between 6 weeks and 6 months earlier. Three serial intranasal passages of lung suspensions and three intraperitoneal passages of spleen suspensions were made, both normal and recovered mice being used for each passage. The results were completely negative.

Attempts to demonstrate the persistent presence of virus in the livers and spleens of 15 mice which had recovered from infection were negative when the mice were tested more than 5 weeks after being inoculated with virus. It is unlikely that this was due to the masking effect of neutralizing antibody, for virus had been successfully isolated by intraperitoneal inoculation from the livers and spleens of mice infected 4 weeks earlier, although their E-AHA titres were very high (1280 and 2560). Further, dilution and high speed centrifugation of the spleen and liver suspensions failed to reveal " masked " virus.

It has been pointed out previously (Fenner, 1947*c*), and subsequent experience has confirmed the impression, that once mice have recovered from infection they are not infective to cage mates. However, we have not made enough detailed observations on mice for long periods of time to be able to exclude the possibility of occasional cases of relapsing infection. Indeed, there is some evidence that such cases do occur. Ectromelia virus was isolated twice from the swollen but not ulcerated feet of mice which had completely recovered from inoculation in that foot 2 and 7 months earlier, and had not shown evidence of recurrent infection until a few days before sacrifice. Greenwood *et al.* (1936) describe a mouse in which lesions of the feet were observed upon three occasions at intervals of 3 and then 6 months, and the last attack ended fatally. These cases almost certainly represent relapsing infection rather than new infections.

While virus may sometimes persist for months, and clinical relapses may occasionally occur, it seems much more likely that in Kikuth and Gönnert's experiments a mouse suffering from subclinical or mild mouse-pox and harbouring the virus in its organs was included in one of the pools of lungs or feet used at some stage (not necessarily the first) during the experiment. The use of mouse-pox-immune mice for subinoculation, though it might render results somewhat irregular, would not preclude the recognition of the virus in this way, for we have found that animals recently recovered from infection will succumb to the subsequent inoculation of moderately large doses of virus, especially if the intranasal route is used.

We disagree, therefore, with Kikuth and Gönnert's contention that ectromelia virus is a more or less harmless ubiquitous symbiont of mice, resembling herpes simplex in humans in its persistence and in the periodic fluctuations in the host-parasite relationship. Our experimental evidence, and that of Greenwood *et al.* (1936), indicates that the enzootic behaviour of mouse-pox is like that of the other mammalian pox viruses—that is, infection is almost invariably followed by durable immunity which results in complete suppression of the virus. It is not denied that this active immunity may wane, and that a mouse may eventually become susceptible to natural reinfection, but this is probably rare and occurs only after a long interval.

SUMMARY.

Mouse-pox (infectious ectromelia of mice) is the murine representative of the mammalian pox diseases. Natural infection of mice occurs through small abrasions of the skin, producing a primary lesion at the site of entry of the virus after an incubation period of 6·8 ± 1·7 days with the "Moscow" strain and 8·1 ± 1·4 days with the "Hampstead" strain. Mice which survive the acute infection usually develop an extensive rash,—the secondary rash. Virus is eliminated from the infected mouse principally in the discharges from the skin lesions.

Epidemics of mouse-pox occurring after the introduction of 15 infected mice to groups of 100 normal male mice were observed. With the more virulent "Moscow" strain 50 per cent of the exposed mice showed evidence of infection by the 17th day, about 12 days after first exposure to infective animals, and all showed evidence of infection by the 22nd day. About half the mice died, and in the majority of the survivors an extensive skin eruption occurred. This had healed in all surviving animals by the 33rd day, when the experiment was terminated. The "Hampstead" strain was less infectious as well as less virulent, and observation of primary and secondary skin lesions indicated that there was a primary wave involving 60 per cent of the contact animals occurring between the 12th and the 20th days, followed by a secondary series of cases between the 22nd and the 28th days. The mortality rate in this epidemic was 15 per cent.

Investigations of antihaemagglutinin titres in mothers and young showed that some antibody passed through the placenta, but a much larger amount was transmitted in the milk. The antihaemagglutinin titres were a good index of immunity, and young mice which had suckled mouse-pox immune mothers survived 1000 times the dose of virus which killed all mice of the same age suckled by normal mothers.

Serological survey of 13 batches of wild mice from different parts of Victoria showed no evidence of mouse-pox infection in that State.

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